Examining the Effects of Cytoplasmic Tail Splicing Event in Goldfish (*Carassius auratus*) Leukocyte Immune-type receptors

by

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A thesis submitted in partial fulfilment of the requirements for the degree of

Master of Science

in

Physiology, Cell, and Developmental Biology

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ABSTRACT

The innate immune system is important in clearing infections and maintaining homeostasis. Across vertebrates, innate immunity is capable of executing potent effector responses through the actions of highly specialized cells. Effector responses, such as phagocytosis and cytokine secretion, are mediated by the actions of conserved and specialized immunoregulatory receptors. The transduction of extracellular events across the cell membrane by immune receptors allows for the fine-tuning of innate effector responses. The evolutionary conserved nature of these receptors across vertebrates bridges the gap to understanding vertebrate immunity by providing an opportunity to study such processes within a range of model systems of teleost fishes.

In 2006, a novel and diverse family of leukocyte immune-type receptors (LITRs) in channel catfish (*Ictalurus punctatus*) was identified (Stafford et al. 2006). Phylogenetic studies and sequence analyses suggest that LITRs belong to the immunoglobulin superfamily (IgSF) and that they are related to mammalian Fc receptors (FcRs), FcRLs and other Ig-type proteins (Wang et al. 2020). IpLITRs contain both stimulatory and inhibitory types of receptors that influence the functions of innate immune cells through their cytoplasmic tail (CYT) regions. This results in both classical and distinct intracellular signalling pathways. Past research on different types of IpLITRs in our lab has enabled us to investigate how immune receptors interact with adaptor signalling molecules, form both homo- and heterodimers, and regulate cell cytotoxicity using both classical and unconventional inhibitory components. Additionally, we have studied how these receptors differ in capturing and engulfing targets, and how they use unique mechanisms to interfere with the phagocytic response.

Although the catfish model has provided valuable insights into immunoregulatory receptor types in teleosts, our lab has recently focused on exploring other teleost models that present a variety of LITR proteins to study the intricacies of immunoregulation in early vertebrates. Utilizing a transcriptome developed from goldfish spleen tissue, our lab identified 37 unique CaLITRs, each characterized by their own specific extracellular and intracellular domain configurations. Two LITRs of interest are the goldfish (*Carassius auratus*) CaLITR 2.0 and CaLITR 2.1 forms. These receptors are identical except for a 29-amino acid deletion in the CaLITR2.1 CYT region that contains a tyrosine (Y) motif potentially involved in cell signalling. This deletion is presumably due to alternative splicing of the goldfish LITR 2 transcript, and it may play functional roles in the fine-tuning of immune effector functions. To investigate this, CaLITR 2.0 and 2.1 were individually cloned into pDisplay vector, then expressed on the cell surface of mammalian AD293 cells with an N-terminal hemagglutinin (HA)-epitope tag. Latex yellow-green (YG) beads opsonized with anti-HA mAb were then used to stimulate the transfected cells. Finally, their phagocytic activities are examined using an ImageStream X flow cytometer.

Due to the inability to express native CaLITR constructs in AD293 cells, chimeric constructs (C-CaLITR) composed of the extracellular and transmembrane domains of the positive control, IpLITR 2.6b, and the CYT region of CaLITR 2.0 or CaLITR 2.1, were created and used in the phagocytosis assays. C-CaLITR 2.0 showed significantly lower phagocytic activity than the splice variant, C-CaLITR 2.1. This observation was consistent when cells were stimulated with 4.5 μm or 2.0 μm beads, as well as increasing antibody concentration to opsonize bead targets. The CYT region-splicing event removes an immunoreceptor tyrosine-based inhibitory motif (ITIM) motif, which generally inhibits immune responses, leaving only an

immunoreceptor tyrosine-based switch motif (ITSM) in the CYT region of CaLITR 2.1. ITSMs are known to have either inhibitory or stimulatory effects on cell signalling, depending on factors such as the cell type, the intracellular adaptor molecules available, and the co-stimulation of other receptors expressed by the same cell. My results suggest that i) removal of the ITIM amplifies the phagocytic response of C-CaLITR 2.1, and 2) an ITSM-containing receptor can uniquely mediate phagocytosis. The underlying signalling components of C-CaLITR 2.1-mediated phagocytosis involve actin, PI3K, SFK and Syk signalling pathways, which were preliminarily investigated using pharmacological inhibitors. Overall, my results show the first evidence of how CYT region-targeted splicing events can fine-tune immunoregulatory receptor functional potentials.

PREFACE

This thesis was the original work of Hussain Al-Rikabi. No animals were used during the completion of this research; therefore, no ethics committee approval was required. I was responsible for data collection and analysis, as well as for writing this manuscript. Dr. James Stafford contributed philosophical, conceptual, and technical guidance and edited this thesis. I supervised one undergraduate student: Mikayla Pichonsky (BIOL 499).

ACKNOWLEDGMENTS

First, I'd like to thank my family for supporting me throughout this journey. Especially my dad and mom, who worked so hard for decades just so I could get the best education. I'd like to thank my amazing sister, who, regardless of the physical distance, always made sure to check on me. Also, I'd like to thank my two amazing brothers, who inspire me with their kindness and resilience. I could not have finished this stage of my education without having a loving and caring family. Thank you!

I would like to say thank you to my supervisor, Dr. James Stafford, for being the best mentor anyone could ask for. I really appreciate all your help, not just in terms of giving me feedback and suggestions for my work but also for being available to talk about life, food, sports and how Jackson has achieved another developmental milestone!

For my lab colleagues and friends. Words cannot describe how much I appreciate you. Jacob, you showed me the ropes and turned an undergraduate with no lab experience into someone who can pipette (wow! I know, right) and question all his data like a true scientist. Thank you for that! Kendra, thank you for making the lab enjoyable, and for being a supportive friend. My downs would have been worse without your presence, and my ups were always celebrated, thanks to you! Nora, thank you for carrying the lab on your back, but more importantly, thank you for always bringing snacks. Those kept my brain functioning in the long hours of work. Rikus, thank you for being patient while training me, I realize that was not an easy job (Phew!). Sunanda, thank you for being a great and supportive friend. You never hesitated to provide advice whenever I sought your wisdom. Najia, I appreciate the great energy you bring to the room, and I wish you the best in carrying the load of LITR research. Kareem and Samuel, time did not allow me to get to know you more, but I appreciate you for making the lab social events more fun with your unique personalities. To my colleagues in the department, Mirza, RJ, Mikayla, Emilie, Reema, Matthew, Amro, Farah, Enezi, and Ximena, I really enjoyed my time with you. Our conversations were never dull, and picking your brain on science was always fruitful. Thank you all!

Finally, I'd like to thank my amazing partner Renea Zatriqi, who stood by my side throughout this whole journey. You never had a doubt in me, even when I doubted myself. Your faith kept me going. Thank you from the heart! Te dua shum!

TABLE OF CONTENTS

CHAPTER III

MATERIALS AND METHODS

CHAPTER IV

EXAMINATION OF GOLDFISH LITR-MEDIATED CONTROL OF THE PHAGOCYTIC RESPONSE

CHAPTER V

PHARMACOLOGICAL EXAMINATION OF CALITR-MEDIATED PHAGOCYTOSIS

CHAPTER VI

GENERAL DISCUSSION AND FUTURE DIRECTIONS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

- ADCC Antibody-dependent cellular cytotoxicity
- AF Alexa Fluor
- AMPs Antimicrobial peptides
- Arp2/3 Actin-related protein 2/3 complex
- BCR B cell receptor
- BLAST Basic local alignment search tool
- BSA Bovine serum albumin
- C3 Complement component 3
- CCM Cell culture media
- CBP CREB binding protein
- Cbp Csk binding proteins
- CD Cluster of differentiation
- Cdc42 Cell division control protein 42
- cDNA Complementary DNA
- CEACAM Carcinoembryonic antigen-related cell adhesion molecules
- CR Complement receptors
- CREB Cyclic-AMP response element binding protein
- CSF-1 Colony-stimulating factor-1
- Csk C-terminal Src kinases
- CLR C-type lectin receptor
- Co-IP Co-immunoprecipitation
- CTLs Cytotoxic T cells
- CYT Cytoplasmic tail
- CytoD Cytochalasin D
- D Domains
- DAG Diacylglycerol
- DAMPs Danger-associated molecular patterns
- DMEM Dulbecco’s modified eagle media

- DMSO Dimethyl sulfoxide
- DPBS Dulbecco's phosphate-buffered saline
- DrLITR *Danio rerio* leukocyte immune-type receptor
- dsRNA Double-stranded RNA
- EAT-2 EWS/FLI1 activated transcript 2
- EDTA Ethylenediaminetetraacetic acid
- ERK Extracellular signal-regulated kinase
- EST Expressed sequence tag
- F Phenylalanine
- FACS Fluorescence-activated cell sorting
- F-actin Filamentous actin
- FBS Fetal bovine serum
- Fc Fragment crystallizable
- FcR Fragment crystallizable receptor
- FcRL FcR-like
- FSC Forward scatter
- Fwd Forward
- Fyn Src/Yes-related novel oncogene homolog
- G418 Geneticin
- Gab2 Grb2-associated binders
- Grb2 Growth factor receptor-bound 2
- GEFs Guanine nucleotide exchange factors
- GI Gastrointestinal
- HA Hemagglutinin
- HEK Human embryonic kidney
- HLA Human leukocyte antigen
- HMGB1 High-mobility group box 1 protein
- HSPs Heat-shock proteins
- ICAM-1 Intracellular adhesion molecule 1
- IFN Interferon

- Ig Immunoglobulin
- IgSF Immunoglobulin superfamily
- iTAM Inhibitory ITAM
- IL Interleukin
- IP Immunoprecipitation
- IP3 1,4,5-trisphosphate
- IpLITR Ictalurus punctatus leukocyte immune-type receptor
- IRAK Interleukin-1 receptor-associated kinase
- IRF Interferon regulatory factors
- IRGs Immunity-related GTPases
- ISGs Interferon-stimulated genes
- ITAM Immunoreceptor tyrosine-based activation motif
- ITIM Immunoreceptor tyrosine-based inhibitory motif
- ITSM Immunoreceptor tyrosine-based switch motif
- JAK Janus kinase
- JNKs C-Jun N-terminal kinases
- KIRs Killer cell immunoglobulin-like receptors
- LEAP Liver-expressed antimicrobial peptide
- LFA-1 Lymphocyte function-associated antigen 1
- LILRs Leukocyte Immunoglobulin-like receptors
- LITRs Leukocyte immune-type receptors
- LPS Lipopolysaccharides
- LRC Leukocyte receptor complex
- Lyn Lck/Yes-related novel protein tyrosine kinase
- mAb Monoclonal antibodies
- MAC Membrane attack complex
- MAPK Mitogen-activated protein kinases
- MBSU Molecular Biology Service Unit
- MBL Mannose-binding Lectin
- MD-2 Myeloid differentiation factor 2

- MDA-5 Melanoma differentiation-associated protein 5
- MEK Mitogen-activated protein kinase kinase
- MHC Major histocompatibility complex
- MLC Mixed leukocyte cultures
- MYD88 Myeloid differentiation primary response 88
- Nck Non-catalytic region of tyrosine kinase
- NFAT Nuclear factor of activated T cells
- NF-κB Nuclear factor kappa B
- NITRs Novel immune-type receptors
- NK Natural killer
- OAS 2,5-oligoadenylate synthetase
- pAb Polyclonal antibodies
- PAMPs Pathogen-associated molecular patterns
- PBLs Peripheral blood leukocytes
- PBS Phosphate-buffered saline
- PCR Polymerase chain reaction
- PD-1 Programmed cell death 1
- PD-1L Programmed cell death 1-ligand
- PDK Phosphoinositide-dependent kinase 1
- PE Phycoerythrin
- PEACAM-1 Platelet endothelial cell adhesion molecule-1
- PFA Paraformaldehyde
- PFKs Protein tyrosine kinases
- PH Pleckstrin homology
- PI(3,4,5)P2 Phosphatidylinositol 4,5-trisphosphate
- PI(4,5)P2 Phosphatidylinositol 4,5-bisphosphate
- PI3K Phosphoinositide 3-kinase
- pIgR Polymeric immunoglobulin receptor
- PKB Protein kinase B
- PKC Protein kinase C

- PKMs Primary kidney macrophages
- PKNs Primary kidney neutrophils
- PKR protein kinase dsRNA activated
- PLCγ Phospholipase C gamma 1
- PMA Phorbol myristate acetate
- PRRs Pattern recognition receptors
- pSHP-2 Phosphorylated SHP-2
- pSyk Phosphorylated Syk
- Rac Ras-related C3 botulinum toxin substrate
- RBL Rat basophilic leukemia
- RIG-1 Retinoic acid-inducible gene I
- ROS Reactive oxygen species
- RT-PCR Reverse transcriptase PCR
- Rvs Reverse
- SALT Skin-associated lymphoid tissue
- SAMPs Self-associated Molecular Patterns
- SAP SLAM-associated protein
- SFK Src-family kinases
- SH2 Src-homology 2
- SH2D1A SH2 domain protein 1A
- SH3 Src-homology 3
- SHIP SH2-containing inositol 5'-phosphatase
- SHP SH2-containing protein tyrosine phosphatase
- SIgA Secretory IgA
- SIRPα Signal Regulatory Protein α
- SLAM Signalling lymphocytic activation molecule
- Src Sarcoma viral oncogene homolog
- SSC Side scatter
- STAT JAK-signal transducer and activator of transcription
- Syk Spleen tyrosine kinase

- TCR T cell receptor
- TGF-β Transforming growth factor β
- TLRs Toll-like receptors
- TLT-1 Triggering Receptor expressed on Myeloid cells-like transcript 1
- TM Transmembrane
- TNF-α Tumor necrosis factor-α
- TRIF TIR domain-containing adaptor inducing interferon-β
- TREM Triggering Receptor expressed on Myeloid cells
- WAVE WASp family verprolin-homologous protein
- Y Tyrosine
- YG Yellow-green
- ZAP-70 Zeta-chain-associated protein kinase 70

CHAPTER I

INTRODUCTION

1.1 Overview

The innate immune system plays a vital role in recognizing microbes, triggering antimicrobial responses, initiating inflammation, assisting in tissue repair, and maintaining homeostasis. Innate immunity also represents the first line of cellular defence consisting of many specialized cells (e.g., neutrophils, macrophages, and dendritic cells), and their associated receptors (Kanneganti, 2020). Across vertebrates, the host's immune system can execute potent effector responses through the actions of various leukocytes or white blood cells. Innate immune cell effector responses include respiratory burst, nitric oxide (NO) production, NETosis, encapsulation/granuloma formation, phagocytosis, degranulation, cytotoxicity, and cytokine/chemokine secretion, which are activated through the coordinated actions of conserved immunoregulatory receptor-types. Fish, amphibians, birds, and many invertebrate models have all been identified to contain evolutionary conserved innate immune receptor-types and associated intracellular signalling molecules critical for the detection and destruction of pathogens (Buchmann, 2014). For instance, immune cells can discriminate self (host) from non-self (pathogens) by utilizing receptors that bind conserved pathogen-associated molecular patterns (PAMPs) that are unique to each pathogen, or self-associated molecular patterns (SAMPs) that are unique to the host (Hato & Dagher, 2015). When a receptor binds to its ligand, it recruits other relevant receptors and a multitude of kinases, phosphatases, adaptors, and other signalling molecules to establish a network that leads to either activation or inhibition of cellular responses.

In vertebrates, elaborate networks of activating and inhibitory receptor types (e.g., Fragment crystallizable receptors (FcRs), Fc receptor-like (FcRLs), Killer cell immunoglobulin-like receptors (KIRs), Leukocyte immunoglobulin-like receptors (LILRs) and Mannose-binding lectin (MBL)) exist on the surface of immune cells to regulate cellular effector responses (Zhang et al., 2017; Mnich et al., 2020). Most of these receptors belong to the immunoglobulin superfamily (IgSF) and C-type lectin superfamily and are essential in modulating responses to bacterial and/or viral infections, acute and chronic inflammation, autoimmune disorders, and cancer-killing, to name a few (Mkaddem et al., 2019; Campbell & Purdy, 2011; Zhang et al., 2017). Classically speaking, activating receptors associate with membrane-bound adaptor proteins containing immunoreceptor tyrosine-based activating motifs (ITAMs; Farag et al. 2002). Following receptor engagement, Src homology 2 (SH2) containing kinases, such as spleen tyrosine kinase (Syk), are recruited via their SH2 domain binding to phosphorylated ITAMs (Farag et al. 2002). This, in turn, allows Syk to be auto-phosphorylated and activated to further phosphorylate downstream signalling molecules. Numerous outcomes can result from ITAM-dependent Syk recruitment and activation, such as proinflammatory gene expression, antimicrobial enzyme activations, cytokine secretion, and the activation of the Actin-related protein 2/3 complex (Arp2/3) that plays a role in actin polymerization and membrane remodelling required for target engulfment during the phagocytic process (Velle & Fritz-Laylin, 2020). In contrast, inhibitory receptors contain one or more immunoreceptor tyrosine-based inhibition motifs (ITIMs; Billadeau & Leibson, 2002). When inhibitory receptors are ligated, spleen family kinases (SFKs) phosphorylate the ITIM, which subsequently recruit tyrosine phosphatases (e.g., SH2-containing protein tyrosine phosphatases (SHP-1), (SHP-2), and SH2-containing inositol 5'-phosphatase (SHIP)) to dephosphorylate intracellular signalling mediators

(Billadeau and Leibson, 2002). Inhibitory receptors help regulate cellular activation through various means, including the recruitment of C-terminal Src kinases (Csk) to Csk binding proteins (Cbp) that phosphorylate residues on SFKs; resulting in their deactivation (Tourdot et al., 2013). Alternatively, inhibition can also be attributed to ITIM tyrosine phosphorylation and SH2 domain-based binding of tyrosine phosphatases (SHP-1) and inositol phosphatases (SHIP) to tyrosine residues within the ITIM (usually in tandem), which facilitates the dephosphorylation of activated molecules (Motoda et al., 2000; Tourdot et al., 2013). Studies also suggest that ITAMs can exert cellular inhibition while ITIMs may conversely have activating effects under the right conditions, which is in stark contrast to how these motifs were classically defined (Pfirsch-Maisonnas et al., 2011). For example, low avidity binding of IgA to the ITAM-containing FcαRI causes its co-translocation to membrane lipid rafts with the inhibitory SHP-1 molecule. When activating receptors are subsequently bound, they end up in the same location as FcαRI and SHP-1 and are then transported to an inhibitory cell compartment known as the inhibisome. Therefore, ITAM-mediated signals seem to manipulate lipid raft activating capabilities, where signalling molecules are deactivated by SHP-1 (Ivashkiv, 2011). On the other hand, the ITIM-containing receptor, triggering receptor expressed on myeloid cells (TREM)-like transcript 1 (TLT-1) is found on platelets and megakaryocytes and was shown to significantly increase FcϵRI-driven calcium signalling resulting in cell activation. This function relies on the ITIM's ability to attract SHP-2, and when this motif is mutated, recruitment of SHP-2 is diminished, and FcϵRI-driven calcium signalling via TLT-1 co-stimulation decreases (Barrow et al., 2004). TLT-1 does not behave like classical ITIM-containing receptors, which down-regulate calcium responses. In essence, these findings highlight the functional plasticity of immunoregulatory receptors and tyrosine-based signaling motifs.

Mechanisms of receptor-mediated control of innate immune cell effector responses are well-studied in mammals but less so in other animals. Fish, amphibians, birds, and many invertebrate models have all been identified to contain evolutionary conserved innate immune receptor-types and intracellular signalling molecules critical for the detection and elimination of pathogens. In 2006, a novel and diverse family of leukocyte immune-type receptors (LITRs) in channel catfish (*Ictalurus punctatus*) were identified (Stafford et al., 2006). Phylogenetic studies and sequence analyses showed that IpLITRs belong to the IgSF and that they are distantly related to mammalian FcRs and FcRLs (Wang et al., 2020). IpLITRs are polymorphic and polygenic and are expressed by both myeloid and lymphoid cell lines (Stafford et al., 2006; Wang et al., 2020). Over the past decade, a considerable amount of information on the biochemical and functional potentials of IpLITRs has been gained by expressing IpLITRs in well-characterized mammalian cell lines (Fei et al. 2016). For example, IpLITR2.6b (an activating receptor) was shown to associate with the ITAM-containing IpFcRγ, IpFcRγ-L, and IpCD3ζ-L adaptors (Mewes et al., 2009). Subsequently, an IpLITR2.6b/IpFcRγ-L fusion protein was shown to induce ITAM-dependent signalling and cellular effector responses, including degranulation and phagocytosis in a rat basophilic leukemia (RBL) cell line (Cortes et al. 2012). Furthermore, IpLITR2.6b was shown to form homo- and heterodimers with other stimulatory IpLITR-types (Mewes et al. 2009). In contrast, the inhibitory IpLITR1.1b was shown to recruit inhibitory phosphatases, including SHP-1 and SHP-2, following tyrosine phosphorylation of its ITIMs (Montgomery et al. 2009).

By studying catfish LITRs, our lab has been able to draw conclusions about immunoregulatory receptor-mediated regulation of immune cell effector functions that were expected for putative stimulatory and inhibitory receptor-types. However, studying IpLITRs has

also revealed novel mechanisms of innate immune control. For example, the ITIM-containing receptor IpLITR 1.1b was shown to inhibit cellular cytotoxic killing not just through a predicted SHP-1-mediated mechanism but in part due to an unexpected SHP-1-independent mechanism via its tyrosine-containing membrane-proximal region via the recruitment of Csk (Montgomery et al., 2012). When transfected in myeloid rat basophilic leukemia cells (RBL-2H3), IpLITR 1.1b also displayed a novel ITAM-independent mode of phagocytosis, highlighting the functional plasticity of these receptors with inhibitory and stimulatory properties (Cortes et al., 2014; Lillico et al., 2015). While LITRs were first discovered in catfish, they also exist in highly conserved gene clusters in other closely related teleost fish, such as zebrafish (*Danio rerio*) and goldfish (*Carassius auratus*). For example, the goldfish LITR predicted repertoire is diverse and unique from other teleost fish, in terms of their extracellular Ig-domains composition and the arrangement of tyrosine motifs in their CYT region, which is likely due to lineage-specific diversification. Interestingly, specific LITR variants with CYT region modifications (i.e., potentially due to alternative splicing and gene duplication events) were identified in the goldfish LITR repertoire. Such mechanisms are commonly observed in extracellular regions of receptors but less so in CYT regions implicated in cell signalling and, consequently, immunoregulation. Therefore, CaLITRs provide us with a set of receptors that can be used to gain insight into the functional consequences of CYT region modifications naturally occurring in teleost immune-type receptors.

Like IpLITRs, CaLITRs encode extracellular Ig-like domains that are phylogenetically and structurally related to Ig-domains of mammalian FcRs and FcR-like proteins (Want et al., 2021). The configurations of their CYT regions are highly variable and consist of unique sets of ITIMs, ITSMs (immunoreceptor tyrosine-based switch motifs), Nck recruitments motifs and predicted

ITAM-like motifs. Of interest for my project are the splice variants CaLITR 2.0 and 2.1, which are identical except for a 29-amino acid deletion in the CYT region of the latter, which removes a membrane distal tyrosine (Y^{465}) motif potentially involved in cell signalling. This deletion is potentially due to an alternative splicing event and implicates targeted changes in LITR CYT regions for the fine-tuning of immune cell effector functions. To further investigate teleost LITRs that encode unique arrangements of amino acid motifs in their CYT, and to understand how fine-tuning of immunoregulatory receptors at the pre-mRNA level is functionally implicated in immunoregulation, the focus of my project was to clone CaLITR 2.0 and CaLITR 2.1 into mammalian expression vectors, and subsequently transfect them into mammalian cell lines to obtain CaLITR-expressing stable cell populations. Then, using our established ImageStream-based phagocytic assay as a model for examining CaLITR immunoregulatory potential(s), CaLITR-expressing cells were triggered with epitope-specific mAb antibodies, and their phagocytic activity was measured. Additionally, the role of key signalling molecules potentially involved in a predicted phagocytic response was examined using pharmacological inhibition assays.

Initial sequence analysis of CaLITR 2.0 suggested the presence of an ITAM-like motif ([E]xxY^{465}xx[I]$x_{28}$$Y^{494}$xx[L]), displaying a nonconventional spacing of 25 amino acids between the two tyrosine motif pockets. Typically, tyrosines within an ITAM are separated by 6-12 amino acids (Billadeau & Leibson, 2002). Therefore, it was hypothesized that, when expressed on the cell surface of AD 293 cells and engaged with mAb-opsonized beads, CaLITR 2.0 would induce phagocytic activity via an ITAM-like dependent signalling mechanism. The splice variant, CaLITR 2.1, was predicted to result in diminished phagocytic activity compared to 2.0, a consequence of a CYT-directed splicing event removing a sequence of 29 amino acids, including

$Y^{465.}$ This modification structurally discriminates the nearly identical receptors from an ITAM-like-containing one to an ITSM-containing variant. Overall, I, therefore, hypothesize that alternative splicing of tyrosine-containing regions within CYT domains of goldfish LITRs will significantly impact their signalling ability and, consequently, their capacity to modulate immune effector functions.

1.2 Objectives

The main objective of this thesis was to examine the functional differences between the newly discovered alternatively spliced CaLITRs and, specifically, how the splicing event is implicated in immune effector function. The specific aims of my research were: i) generate wild-type CaLITR constructs for the transfection and creation of CaLITR-expressing AD293 cell lines. ii) utilize newly created CaLITR-expressing cells to examine the importance of alternative splicing using flow cytometric phagocytosis assay. iii) characterize the signalling components (and pathways) involved in CaLITR-mediated phagocytosis.

1.3 Outline

Chapter II is an overview of literature relevant to the topics covered in this thesis. In this chapter, I will provide general information on the vertebrate immune system, and its physiological importance, with a specific focus on innate immunity. Then I will review teleost models used to study immunity, and how such models provide an incredible amount of information on receptor-based immunoregulation. Then I will dive deeper into LITR-based studies from catfish and zebrafish models and conclude with up-to-date discoveries on goldfish LITRs. Chapter III includes a detailed description of the research techniques, reagents, and methodologies used to complete the research described in this thesis. In Chapter IV, I show and discuss the results of research testing the hypothesis that CYT-region splicing of the ITAM-like-

in CaLITR 2.0 fine-tunes the phagocytic phenotype observed by the ITSM-containing CaLITR2.1 This was achieved using CaLITR-expressing AD293 cell lines and flow cytometric phagocytosis assays. My results show that CaLITR 2.1 induces a stronger phagocytic response than CaLITR 2.0 when engaged with either small (2 μm) or large (4.5 μm) bead targets. In Chapter V, I investigated the general signalling components involved in the different phagocytic responses observed between CaLITR 2.0 and CaLITR 2.1. This was done using pharmacological profiling of key signalling pathways. These observations suggest that the ITSM-containing CaLITR 2.1 facilitates phagocytosis in actin, phosphoinositide 3-kinase (PI3K), Src-family kinases (SFK)-dependent manner and, to a lesser degree, may involve Syk activity. In conclusion, chapter VI summarizes all the research findings in this thesis on how CaLITRs 2.0 and 2.1 expand our knowledge on immunoregulatory control of cellular effector responses, as well as future directions for this project. Chapter VII includes a bibliography of all the references used in this thesis.

CHAPTER II

LITERATURE REVIEW

2.1 Innate immunity

The immune system is extremely important in protecting the host from pathogens and maintaining body homeostasis. It consists of complex defence mechanisms that have evolved to counter the many ways foreign agents can inflict damage. These mechanisms include physical anatomical barriers, tissue-specific immune cells, receptor-mediated cellular functions, humoral factors, and adaptive and memory cells for more specific and configured responses (Chaplin, 2010). The immune system is divided into two main branches: innate immunity, which comprises the anatomical physical barriers, and cellular and humoral components, which recognize conserved features of pathogens to launch an immediate (within minutes to hours) response. In contrast, adaptive immunity provides a long-lasting defence that requires an intricate and controlled process (1-2 weeks) of immune cell training following the initial pathogen recognition. Adaptive immune cells (T and B cells) are more specific to the encountered pathogen and, once recruited to the site of infection, can execute a rapid effector response. Additionally, following secondary exposures, T and B cells produce a stronger effector response through the preservation of memory cells in lymphoid tissues (Chaplin, 2010). For example, memory B-cells become activated upon stimulation by an antigen previously encountered, which consequently leads to the proliferation and differentiation into specialized, immunoglobulin-secreting lymphocytes known as plasma B cells (Cyster & Allen, 2019). Antibodies (immunoglobulin-based proteins) contain a variable region that recognizes specific molecular sequences of antigens, such as those on the surface of pathogens, while also containing a fragment crystallizable (Fc) region recognized by immune cells through receptors on their surface (Cyster & Allen, 2019).

While the adaptive immune system provides a potent, lasting defence against recurring infections, the innate immune system is vital as the initial responder against the preliminary phases of microbial attack (Chaplin, 2010). The skin and mucosal layers are well-studied physical barriers that provide anatomical blockade to pathogen invasion. When this barrier is breached, tissue-resident innate immune cells are activated to launch a proinflammatory attack and simultaneously send recruitment signals to other immune cells. Neutrophils, macrophages, and dendritic cells comprise the arms of first responders at the site of infection (Nguyen & Soulika, 2019). These cells are equipped to sense and detect pathogens through the expression of germline-encoded pattern recognition receptors (PRRs) that bind to pathogen-associated molecular patterns (PAMPs). This PRR-PAMP interaction may then result in an effector function such as phagocytosis, degranulation, and cytokine secretion. The magnitude and the type of effector response are contingent on the type of ligand bound, the type of receptor initiating the signalling cascade, and the immune cell(s) involved (Nguyen & Soulika, 2019). As an example, bacterial lipopolysaccharide (LPS) bound to macrophages-expressed toll-like receptor 4 (TLR4) triggers signalling cascades that culminate in the production of proinflammatory cytokines such as Tumor Necrosis Factor α (TNFα), Interleukin-1β (IL-1β), Interleukin-6 (IL-6), and Interleukin-12 (IL-12) (Akira & Takeda, 2004). TLRs are highly conserved pattern recognition receptors, originally discovered in *Drosophila melanogaster*, and have homologues expressed in a wide range of organisms, including *C. elegans*, lamprey, chickens, and humans (Kuzmich et al., 2017).

The skin insulates the body's interior environment from the pathogen-filled exterior environment (Nguyen & Soulika, 2019). It achieves this through the outermost layer, the epidermis, which consists of several tiers of tightly packed epithelial cells connected by tight

junctions that prevent pathogen entry. If the epidermis is breached, the inner layer of the skin, the dermis, which contains blood vessels, secretory glands, and immune cells of myeloid and lymphoid lineage, can initiate an immediate cellular response to stop pathogen infiltration (Kaplan, 2017). In addition to the skin, epithelial layers also cover tissue surfaces exposed to the outer environment, such as the gastrointestinal tract and the respiratory tract. While the overall composition can differ, all vertebrates secrete a form of lubricant to help maintain the overall integrity of the external layers. In fish and amphibians, this layer takes the form of a mucosal membrane composed of glycoproteins and monosaccharides, while terrestrial animals secrete a lipid and glycolipid-based fluid (Kueneman et al., 2013). In conjunction with providing physical protection, epithelial cells secrete a broad spectrum of proteins and peptides that constitute the chemical arms of innate defence. One well-studied family of molecules is the antimicrobial peptides (AMPs); cationic molecules with hydrolytic capabilities against bacteria, viruses, and fungi (Niyonsaba et al., 2017). First discovered in frogs, antimicrobial peptides were also shown to have essential antimicrobial functions in mammals, birds, fish, and plants. An example of AMPs is lysozyme (muramidase or N-acetylmuramichydrolase), which cleaves the peptidoglycan structural units in gram-positive bacteria, resulting in bacterial cell death (Niyonsaba et al., 2006).

While non-specific physical and chemical host defences provide protection against pathogen invasion, microbes have evolved mechanisms to enter host tissues, and consequently, more sophisticated levels of host cellular and molecular defence mechanisms are needed. Such mechanisms rely on an integrated system of sensors, transducers, and effectors to work effectively (Chaplin, 2010). The sensors in this system are immune receptors (e.g., PRRs) that recognize and bind to foreign molecules. Upon receptor-ligand interaction, signalling-associated

transducers such as protein tyrosine kinases (PFKs), guanine nucleotide exchange factors (GEFs), and Rho family GTPases culminate the extracellular-induced signal towards an intracellular effector molecule, such as the nuclear factor kappa B (NF-κB). NF-κB is a conserved transcription factor best known for mediating immune effector functions such as the production of pro-inflammatory cytokines (Liu et al., 2017). For example, TLR-5 is a pattern-recognition receptor that is expressed on the cell surface of macrophages, neutrophils, dendritic cells, and epithelial cells. It binds to a conserved sequence on bacterial flagellin, a structure needed for host colonization and motility (Yoon et al., 2012). Upon TLR5-flagellin binding, TLR5 transduces a stimulatory signal through the well-studied Myeloid differentiation primary response 88 (MyD88)/ Interleukin-1 receptor-associated kinase (IRAK) signalling protein complex, which converges to activate NF-κB, and ultimately resulting in the production of proinflammatory cytokines such as IL-12, IL-6 and TNFα (Yoon et al., 2012). Almost all bacteria have flagella, making TLR5-induced immune modulation very important for maintaining homeostasis. While foreign, non-self, ligands are shown to be immunogenic, self-produced molecules can also activate the immune system. Endogenous danger-associated molecular patterns (DAMPS) from damaged, dead, or stressed cells can come from a variety of sources and include molecules such as heat-shock proteins (HSPs), high-mobility group box 1 protein (HMGB1), and S100 proteins (Schaefer, 2014). DNGR-1 (a.k.a. CLEC9A) is expressed selectively by dendritic cells and binds to cells that have lost plasma membrane integrity, specifically F-actin molecules (Cueto et al., 2020). The binding of DNGR-1 to F-actin from damaged cells leads to Src Family Kinase (SFK)-dependent hemITAM phosphorylation and Syk activation, but the initiated signal does not converge to NF-κB and does not induce DC activation. Rather, DNGR-1 signalling appears to regulate endosomal maturation to favour the

presentation of dead cell-associated antigens by the Major Histocompatibility Complex (MHC) class I molecules, a process known as cross-presentation (Cueto et al., 2020). MHC molecules present self or non-self-antigens to specialized immune cells such as Dendritic cells, T cells and NK cells (Wieczorek et al., 2017). While HemITAMs are signalling motifs consisting of the classical tyrosine tandem (i.e., Yxx[I/L/V]), but uniquely preceded by a stretch of three negatively charged amino acids, a tandem known as the tri-acidic motif (Bauer & Steinle, 2017).

Cellular effector responses are important manifestation of the immune system's intracellular circuits. Recognition of PAMPs and DAMPs without a modulatory action is not favourable for the survival of the host. There are two branches of immune effector functions: i) cell-mediated responses and ii) humoral responses (Kirimanjeswara et al., 2008). Cell-mediated responses are initiated through the discrimination of “non-self” or “altered-self” from “self”-expressing cells. PAMPs are the classic non-self molecules, while altered-self includes early and late-stage apoptotic cells (González et al., 2011). When undergoing the process of programmed cell death, those cells downregulate the expression of CD47, which is a ligand found on all healthy cells that binds Signal-Regulatory Protein α (SIRPα) on macrophages, dendritic cells (DC) and Natural Killer (NK) cells. CD47-SIRPα binding recruits inhibitory phosphatases (e.g., SHP-2) to the ITIM-containing cytoplasmic tail of SIRPα; ultimately resulting in the propagation of what is known as “don’t eat me” signals (Morrissey et al., 2020). Therefore, the downregulation of CD47 dampens the “don’t eat me” signals; allowing professional phagocytes to carry out their duty of cellular clearance. On the other hand, humoral effector responses include soluble plasma components such as complement proteins, antibodies, and cytokines. Initially identified for its capacity to enhance the antimicrobial attributes of antibodies, the complement system consists of a sequence of glycoproteins and enzymes present in the serum

and on membranes. These complement components aid in opsonizing and triggering inflammatory and lytic responses against harmful pathogens (Dunkelberger & Song, 2009). The vertebrate complement system can be activated in three ways. The classical pathway is initiated by an antibody-antigen immune complex, formed by IgM or IgG antibodies opsonizing a target pathogen. The lectin pathway is activated by the binding of a protein complex consisting of mannose/mannan-binding lectin in bacterial cells, and the alternative pathway is activated independently of any protein complexes; usually through direct interaction of serum complement proteins to microorganisms (Dunkelberger & Song, 2009). From a molecular lens, complement protein C3 is cleaved into C3b (an opsonin) and C3a, a convergence point for all three complement pathways. C3b acts to identify the opsonized target with a molecular "tag," leading to further complement cascades and assembly of a membrane-penetrating macromolecular pore (membrane attack complex; MAC), lysing the targeted pathogen (Heesterbeek et al., 2019). Overall, humoral and cell-mediated mechanisms are extremely important in the execution of immune responses. And, more often than not, they work together to achieve body homeostasis.

In this literature review, I will talk about the mechanisms by which vertebrates modulate immune responses, with a specific focus on immune receptor-types. Then I will provide an overview of teleost immunity, and how our lab, as well as many other labs, have utilized their intricate systems to better understand the nuances of immunoregulation. Then to give you the full picture of relevant background information for this thesis, I will review the research done on Leukocyte immune-type receptor (LITR) proteins.

2.2 Immunoregulatory receptors

2.2.1 Overview

Cellular responses to pathogens, infected cells or dead cells are contingent on the initial recognition of those entities. Recognition of self, altered self, or non-self is initiated by immune cells expressing repertoires of immunoregulatory receptor-types that allow cells to translate extracellular cues (i.e., ligand binding) into intracellular signalling events controlling immune cell effector responses (Kanneganti, 2020). This intricate system of sensing, signal transduction, and effector response is conserved across eukaryotes, including mammals, plants, fungi, and protists (Buchmann, 2014). Generally, immunoregulatory receptors are specialized proteins expressed on the surface of the plasma membrane that are composed of three distinct regions. The extracellular domain contains the ligand-binding amino acid residues and can be made of leucine-rich repeats (e.g., TLR4), glycoproteins (e.g., mannose-binding lectin) or immunoglobulins domains (e.g., FcRγ) (Roudaire et al., 2021, Akula et al., 2014). The transmembrane (TM) segment anchors the receptor in the phospholipid bilayer and may contain a positively charged (e.g., arginine) amino acid residue to recruit other receptors or signalling-adaptor proteins via electrostatic associations (Saint-Georges et al., 2001). The intracellular cytoplasmic (CYT) region contains one or more tyrosine-based signalling motifs that, once phosphorylated, recruit other signalling adaptors and transmit an intracellular signal resulting in effector functions such as phagocytosis, degranulation, cell-mediated cytotoxicity, and cytokine secretion (Lemmon & Schlessinger, 2010). Described above is the general scheme of how immunoregulatory receptor types are structured, localized, and operate. However, it is worth mentioning that the host-pathogen perpetual war of co-evolving to outlast the other has led to the

evolution of elaborate mechanisms in which immunoregulatory receptors mediate immune responses. In the next section, I will shed light on some of those mechanisms.

2.2.2 Immunoregulatory receptor-mediated Effector Responses.

Neutrophils, dendritic cells, and other immune-type cells strategically position their immunoregulatory receptors in different parts of the cell to respond to different types of pathogens at different stages of target acquisition (Wang & Thomson, 2022). For receptors that recognize and bind to extracellular entities, the cell places them on the surface with their ligand-binding extracellular domain facing the environment where they will encounter PAMPs or DAMPs. This is achieved through post-translational modification in the endoplasmic reticulum (ER) where proteins are tagged with specific signal peptides to be trafficked to the cell surface (Zarei et al., 2004). For instance, dendritic cells express Dectin-1, a member of the C-type lectin receptor (CLR) family, on its cell surface to bind β-1,3-glucan on bacteria and fungi located in the extracellular space. This triggers a series of signalling cascades leading to the activation of NF-κB, mitogen-activated protein kinase (MAPK), and nuclear factor of activated T cells (NFAT) signalling modules (Zarei et al., 2004). DCs activated by Dectin-1 signalling have the capacity to prime T cells, favouring the induction of Th17 cells, a CD4+ effector T-cell type that provides immunity to fungi and extracellular bacteria (LeibundGut-Landmann et al., 2007). In the case of phagocytosed or endocytosed extracellular antigens, immune receptors are trafficked via vesicles to the endosomal membrane, where they can bind pathogen-digested ligands. This amplifies cell activation, triggers phagosome formation and phagolysosome maturation, then ultimately, leads to the production of reactive oxygen species (ROS) that eliminate the engulfed pathogen (Mata-Martínez et al., 2022). A mouse B-cell line, LK35.2, was shown to recruit Dectin-1 to the phagocytic cup, as well as the phagosome, following the binding and engulfment

of β-glucan-containing zymosan (Rogers et al., 2005). This process was seen to occur in conjunction with Syk recruitment to the phagosome, suggesting that endosomal expression of Dectin-1 sustains the activation state of the cell.

While some receptors localize at both the cell surface and the endosomal surface, such as Dectin-1 and TLR3; other receptors are preferentially expressed at the endosomal membrane. TLR8, an endosomal sensor of ssRNA or RNA degradation products, is such an example. TLR8 was shown to recognize viruses, including Influenza, Senday and Coxsackie B (Hu et al., 2018). Viruses are small intracellular pathogens (5 to 300nm) that enter the cell and hijack its replication machinery to spread and cause infection. Once endocytosed, the viral genetic material can be detected using pattern recognition receptors (PRRs), including TLR8 and TLR7 for single-strand RNA-containing viruses and TLR3 for double-strand RNA-containing viruses (Kawasaki & Kawai, 2014). This strategic localization of endosomal PRRs allows the cell to launch an antiviral response via MyD88-engagement and upregulation of NF-κB, interferon regulatory factor 3 (IRF3) and IRF7.

Aside from endosomal compartments, intracellular receptors are also found in the cytosol, such as retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5), NOD1 and NOD2 (Kanneganti, 2020). These receptors detect intracellular pathogens that escape the endosomal compartments, enveloped viruses that fuse their membrane to the host's cell membrane and deposit their genetic material directly into the cytoplasm, or fragments of bacteria remaining inside the engulfed cell. As an example, RIG-I binds to the 5’triphosphorylated, blunt-end termini of viral ssRNA, a unique feature different from the host’s RNA. This results in the induction of type 1 interferons (such as IFNα) and interferon-stimulated genes (ISGs) to establish an overall antiviral state (Kato et al., 2005).

2.2.3 Immunoglobulin Superfamily (IgSF) Proteins

The immunoglobulin superfamily (IgSF) is one of the largest and most extensively studied protein families in vertebrates. Produced as cell surface and/or soluble receptors; they play a central role in cellular processes such as antigen recognition, cell-cell adhesion, and modulating innate and adaptive immune responses (Yap et al., 2014). A common feature among these proteins is the presence of an immunoglobulin domain, a specific protein domain made up of 7-9 antiparallel β-strands that form two β-sheets that consist of roughly 70-110 amino acids. The IgSF encompasses a wide range of molecules, such as antigen receptors on the cell surface (e.g., CD4 and CD8), immune system co-receptors and co-stimulatory molecules (e.g., CD86, CD28), molecules that assist in presenting antigens to lymphocytes (e.g., MHC I and II), cell adhesion molecules (e.g., Intracellular adhesion molecule 1; ICAM-1), certain receptors for cytokines (e.g., Interleukin-1 receptors; IL-1R), and receptors that bind to antibodies or MHC molecules to induce immune responses (e.g., FcRs and Killer Ig-like receptors; KIRs) (Harpaz & Chothia, 1994; Moretta & Moretta, 2004).

Fc-receptors (FcRs) are well characterized across vertebrates (mammals, birds, amphibians, fish) for their capacity to bind to the Fc region of antibodies and trigger modulatory immune responses. For instance, FcγRIII expressed on the cell surface of macrophages binds to IgG-opsonized targets, such as pathogenic bacteria or virally infected cells, to induce robust phagocytosis and ultimately clear those targets (Takai, 2005). Additionally, FcγRIII-IgG binding on NK cells induces them to create a lytic synapse to the opsonized target and sequentially release cytotoxic factors such as perforin and granzymes that lead to cell lysing and, ultimately, cell death (Peppa, 2017). This process is known as antibody-dependent cellular cytotoxicity (ADCC), and it demonstrates how the two arms of innate immunity (e.g., NK cells,

macrophages) and adaptive immunity (e.g., IgG antibodies) can join forces to protect the host. IgE, unlike other isotypes, is mainly found in tissues and plays a key role in managing type I hypersensitivity reactions (Janeway et al., 2001). It does so by binding to the FcεRI receptor, which has a high affinity for IgE antibodies, and upon binding, it induces mast cells to release chemical mediators such as histamine, heparin, and prostaglandin to physiologically resolve the allergen. IgA, on the other hand, is known for its role in maintaining the mucosal layers and can exist in both dimeric and monomeric forms. Though present in serum in low amounts, IgA is primarily detected in mucosal secretions like those from the gastrointestinal tract, tears, sweat, and saliva (Snoeck et al., 2006). When the dimeric form of IgA is synthesized and secreted by plasma cells, it can bind to the polymeric immunoglobulin receptors (pIgR) located on the basolateral side of epithelial cell membranes. The IgA is subsequently transported to the luminal side of the membrane and is released as secretory IgA (SIgA). There, it has the capacity to bind to and neutralize potential threats before they can infiltrate the epithelial cell layer (Bakema & van Egmond, 2011). In addition to tolerance, IgA can also mediate proinflammatory responses through the engagement of FcαRI expressed on macrophages (i.e., to induce phagocytosis), basophils and eosinophils (i.e., to induce degranulation).

Close relatives of FcRs are the Fc-receptors-like (FcRLs) proteins, which are IgSF receptors with various isoforms, FcRLs 1 to 6, clustered closely to FcRs and the SLAM family in the genome of humans, mice, chickens, and frogs (Wang et al., 2021). Due to alternative splicing of the mRNA, FcRLs display a variety of domain structures, tyrosine-containing CYT regions, secretion properties, as well as different expression profiles in different host tissues (Rostamzadeh et al., 2018). For instance, four alternate transcripts encoding different FcRL6 isoforms have been identified, each possessing unique extracellular and/or intracellular domains.

These are labelled as variants FcRL6 v1 through v4. The longest isoform is encoded by FcRL6-v1, whereas the FcRL6-v2 isoform encodes a single extracellular D5 domain, with a CYT identical to that of the FcRL6-v1 isoform, which contains three ITAMs (Rostamzadeh et al., 2018). Additionally, FcRL5 has three isoforms: the secreted form (IRTA2a), the GPI-anchored form (IRTA2b), and the transmembrane form (IRTA2c). Among these, the intact FcRL5 (IRTA2c) is the most complex, containing nine Ig-like domains, whereas the GPI-anchored and secretory forms contain six and eight domains, respectively. Notably, the secreted isoform has been detected in high amounts in patients suffering from multiple myeloma (MM), chronic lymphocytic leukemia (CLL), and mantle cell leukemia (MCL) (Hatzivassiliou et al., 2001). These observations indicate the potential for FcRL5 to serve as a valuable diagnostic marker and possible treatment target for blood cancers. In terms of the cellular function of this family, it was observed that co-ligating of the ITIM and ITAM-containing FcRL3 with the BCR resulted in the suppression of B-cell mediated calcium mobilization, cellular tyrosine phosphorylation, and MAPK activation via the recruitment of SHP-1 and SHP-2 and the subsequent inhibition of Syk and phospholipase C-gamma 2 (PLCγ2). In another context, co-stimulation of FcRL3 and TLR9 augmented TLR9-mediated B-cell proliferation, activation and survival via the NF-κB and MAPK pathways (Li et al., 2013).

The IgSF consists of other related receptor groups, including the killer Ig-like receptors (KIRs). This group displays remarkable diversity both between and within populations; mainly due to its polymorphic nature, a characteristic possibly shaped by evolutionary pressures in response to constantly changing pathogen ligands. KIRs are found on human chromosome 19q13.4, within the human leukocyte receptor complex (LRC). Predominantly expressed by NK cells and T-cells, they bind to antigens presented by the polymorphic human leukocyte antigen

(HLA) class 1 molecules (Harrison et al., 2022; Pascal et al., 2007). For example, KIRD3L1 is an inhibitory receptor containing two ITIMs in its intracellular CYT region. When bound to self-antigens presented by HLA-A or HLA-B molecules, the ITIMs become phosphorylated by SFKs, which subsequently recruit SHP-1 and SHP-2 to the site of receptor engagement. This leads to the dephosphorylation and inactivation of stimulatory intermediates such as Zeta-chain-associated protein kinase 70 (ZAP70), PLC-γ, and LAT (linker for activation of T cells); ultimately suppressing NK cell cytotoxicity (O'Connor & McVicar, 2013). Virally infected or stressed cells lose or alter the expression of HLA-A and/or HLA-B expression, thereby eliminating the inhibitory signals induced by KIR3DL1. Simultaneously, activating receptors such as KIR2DS or KIR3DS can still bind to subsets of HLA-C molecules expressed on those stressed cells, leading to the recruitment of transmembrane-bound ITAM-containing adaptor DAP12, which subsequently gets phosphorylated and activated by SFKs. This leads to Syk recruitment and the activation of MAPK pathways, which ultimately leads to increased NK cell cytotoxicity and the production of proinflammatory cytokines such as interferon-gamma (IFN-γ) (Chen et al., 2020). The balance between inhibitory and activating signals mediated by KIR engagements with different targets regulates the activity of NK cells.

Altogether, studies on various vertebrate model organisms, like mice, birds, and amphibians, reveal that IgSF members cluster in areas like those of the LRC and FcR complexes. As previously mentioned in relation to PRRs, IgSF members are encoded in the germline and can identify a broad spectrum of molecules such as pathogen molecules, opsonins, host proteins, and numerous other ligands, allowing for refined immune cell responses. Both myeloid and lymphoid cell lineages express dynamic arrangements of IgSF receptors, with a balance of activating and inhibitory receptor types ensuring tightly regulated immune effector responses.

2.2.4 Immunotyrosine-based immunoregulatory signaling.

Recognition and binding of ligands by immunoregulatory receptors trigger receptor cross-linking and signal transduction through the intracellular recruitment and activation of various adaptors and effectors; a dynamic process facilitated via specific arrangements of signalling motifs located within the CYT regions of receptors (Janeway et al., 2001). The ensuing effector function and its magnitude are contingent on the type(s) of signalling motif(s) found in the CYT of the bound receptor, the signalling molecules recruited, and the elaborate downstream networks (or cascades) activated. Therefore, the sequence of amino acids in CYT of receptors directly influences immunoregulation. Tyrosine, serine, and threonine are among the most common residues implicated in cell signalling via phosphorylation/de-phosphorylation mechanisms, however, the roles of tyrosines will be the focus of this thesis (Taylor et al., 1995).

Conventionally, immune receptors with short CYT are shown to be stimulatory; inducing effector responses via charged residues in their TM segments that pair with signalling-motif-containing adaptor proteins carrying opposite charges, such as FcRγ and CD3γ (Ben Mkaddem et al., 2019; Torres, 2002). These adaptors contain immunoreceptor tyrosine-based activation motifs (ITAMs; amino acid sequence [D/E]xxYxx[I/L] $x_{(6–12)}$Yxx[I/L]), which become phosphorylated by Src-family kinases (SFK; i.e., Lyn, Fyn) upon receptor engagement. This phosphorylation event occurs at tyrosine residues embedded within the ITAM. Subsequently, kinases that contain Src-homology 2 (SH2) domains, like Syk or ZAP-70, are recruited to the receptor engagement sites via their SH2 domain binding to the dual phosphorylated tyrosines embedded within the ITAMs. This interaction leads to the phosphorylation and subsequent activation of ZAP-70 (or Syk), which can then activate other key signalling molecules. Concurrently, certain proteins, such as PLCγ, convert phosphatidylinositol 4,5-bisphosphate

(PI(4,5)P2) into 1,4,5-trisphosphate (IP3) to engage calcium mobilization and diacylglycerol (DAG) for the activation of MAPK (Ben Mkaddem et al., 2019). While this process could diverge into distinct downstream signalling pathways, hence multiple outcomes, it largely contributes to the induction of cell-mediated responses such as cytokine production and degranulation. Phosphoinositide 3-kinase (PI3K), a commonly expressed kinase in almost all cell types, can also mediate ITAM-dependent effector responses. PI3K has the ability to phosphorylate (PI(4,5)P2) into (PI(3,4,5)P3), creating an anchor point for additional signalling molecules possessing the pleckstrin homology (PH) domain, such as PLCγ and protein kinase B (PKB/Akt), to localize at the site of receptor engagement (Yang et al., 2019). Overall, signalling networks are complex and multifaceted and consist of many key players that are interconnected to convert extracellular signals into effector responses. The intricacy of such a system allows host organisms to fine-tune immunoregulation, and, in the case of ITAM-dependent pathways discussed above, it allows for numerous different outcomes to occur. These outcomes include the secretion of antimicrobial peptides, the production of proinflammatory mediators, the secretion of cytokines and chemokines, and the activation of the Arp2/3 complex, which plays a role in F-actin polymerization and the membrane remodelling necessary for the engulfment of targets through the conserved process of phagocytosis (Velle & Fritz-Laylin, 2020)

ITAM-containing receptors include the BCR-associated Igα and Igβ chains, which are essential for initiating signals leading to B-cell activation, maturity, and proliferation (Billadeau & Leibson, 2002). The CD3 complex of T-cell Receptors (TCR) contains a total of ten ITAMs distributed between the CD3ε chains and the ƛ dimers. T cells in CD3ε-deficient mice fail to undergo normal positive selection, suggesting that ITAM signalling is also essential for T-cell specialization. While most activating receptors contain short CYT and associate with ITAM-

containing adaptor proteins (e.g., NKp30 associating with DAP12) to relay extracellular signals, few contain an ITAM within their own CYT region (Medjouel Khlifi et al., 2022). One such example is FcγRIIA, which was shown to induce ITAM-dependent phagocytosis and the production of pro-inflammatory cytokines upon stimulation with LPS (Mkaddem et al., 2017)

In comparison, immunoregulatory receptors that have long CYT regions usually contain unique amino acid configurations with the capacity to result in a variety of functional outcomes. For instance, inhibitory receptor-types represent one such variation, typically containing one or more immunoreceptor tyrosine-based inhibitory motifs (ITIM; amino acid sequence: [S/I/V/L]xYxx[I/L/V]) (Billadeau & Leibson, 2002). These receptors help regulate and dampen the cellular activation induced by its activating counterparts. Although activating receptors are needed to activate immunity, their continuous (i.e., uncontrolled) activation can lead to excessive tissue damage (Chen et al., 2017). Therefore, inhibitory receptors play a crucial role in restoring and maintaining body homeostasis.

Mechanisms of inhibition vary and include the recruitment of phosphatases such as SHP-1/SHP-2 and inositol phosphatases (SHIP) by tyrosine residues located within the ITIM of receptors CYT region. Upon activation, those phosphatases become capable of dephosphorylating activated downstream molecules that were initially contributing to sustaining the activation signal (Tourdot et al., 2013; Motoda et al., 2000). Other methods include recruiting C-terminal Src kinases (Csk) to Csk binding proteins (Cbp) that phosphorylate residues on SFKs, making them non-functional. Inactive SFK cannot phosphorylate ITAMs on activating receptors, therefore, dampening the activation signal. A typical instance of ITIM-mediated inhibition is seen in the innate immune response orchestrated by NK cells, which differs from the FcγR ITAM-dependent ADCC reaction. NK cells inspect for irregular cell phenotypes, such as cells

that are virally infected or apoptotic cells, through the engagement of ITIM-containing KIRs to germline-encoded MHC class I molecules that exist on the surface of all nucleated cells (Chen et al., 2020). This interaction of ITIM-containing receptors facilitates tolerance towards normal cells, preferentially targeting and eliminating altered cells exhibiting abnormal or diminished MHC class I expression profiles, often associated with viral infections and cancers. The MHC molecules present on healthy cells surfaces serve as the ligand for inhibitory-type KIRs, hindering cell-mediated cytotoxicity and destruction. Notably, virally infected cells often employ the strategy of downregulating the MHC class I molecule on their surface, a move designed to dodge the immune response of cytotoxic T cells (CTLs). This evasion strategy prevents the interaction between the MHC molecule, which presents viral peptides, and the T cell receptor (TCR) that would result in the induction of cytotoxic effects. Beyond KIRs, inhibitory motifs are also found in other types of receptors. For example, the FcγRIIB variant stands as the only FcγR known to contain an ITIM within its CYT region (Ben Mkaddem et al., 2019). This receptor has been shown to weaken the activating signals of other FcγRs and B cell receptors (BCRs) by co-ligating with stimulatory receptors through the binding of antibody complexes. This clustering of receptors raises the activation threshold of receptor-based signalling, which leads to a general weakening of cell activation, inhibition of B cell antigen presentation, and even B cell apoptosis when FcγRIIB is engaged in the absence of BCRs (Pearse et al., 1999).

While ITAM or ITIM-containing receptors are more extensively studied, there exist other immunoregulatory receptors with unique amino acid configurations that also play important immunological roles. For example, immunoreceptor tyrosine-based switch motifs (ITSM; amino acid sequence: TxYxx[I/L/V]) were shown to modulate immune functions in either a stimulatory or inhibitory fashion, largely due to the cell context in which the ITSM-containing receptor is

expressed (i.e., different cell types, adaptor proteins, etc.) (Chemnitz et al., 2004). Programmed cell death-1 (PD-1) is a receptor that inhibits T-cell responses upon binding to its ligand, PD-lL, which is considered a critical checkpoint for cancer immunotherapy. When expressed in Jurkat-T-cell lines, the $ITSM^{-/-}$ PD-1 inhibitory capacity was diminished, as measured by high IL-2 (pro-inflammatory cytokine) levels produced in co-cultured B-cell lines (Patsoukis et al., 2020). In comparison, cells containing the wild-type PD-1 were able to significantly inhibit IL-2 production. Furthermore, C-type lectin-like receptors (CTLRs) such as DNGR-1 and Dectin-1 contain a unique signalling motif that resembles the ITAM sequence but with only one tyrosine in the binding pocket, referred to as the hemi-immunoreceptor tyrosine-based activation motif (HemITAM; amino acid sequence: DEDGYxxL). Upon binding of β-glucan to DNGR-1 on the surface of DCs, the HemITAM becomes phosphorylated by SFKs. This receptor exists in dimers due to a disulphide bond linkage, which is a confirmation that allows for the docking and activation of Syk at the phosphorylated HemITAMs via its N-terminal and C-terminal SH2-containing domains. The activation of Syk triggers DC intracellular pathways facilitating the cross-presentation of dead cells-associated antigens (i.e., exogenous antigens) by MHC I molecules (Cueto et al., 2020). Cross presentations prime cytotoxic CD 8+ T cells to launch an all-out enzymatic attack on the foreign entity for complete elimination.

2.2.5 Functional Plasticity

Immunoregulatory receptors are typically categorized based on the presence of immunoreceptor tyrosine-based motifs within their CYT region; ITAM-containing receptors are considered stimulatory, while ITIM-containing receptors are inhibitory. Nevertheless, the resultant effector responses from engaging these ITAM or ITIM-bearing immune receptors don't always follow the traditional patterns of activation or inhibition linked with these classical motifs

(Blank et al., 2009). Indeed, many receptor-types with ITAM or ITIM signalling motifs demonstrate functional plasticity, wherein the same receptor exhibits both inhibitory and activating capabilities. For example, low avidity binding of IgA to the ITAM-containing FcαRI causes its co-translocation to membrane lipid rafts with the inhibitory SHP-1. When activating receptors are subsequently bound, they end up in the same location as FcαRI and SHP-1 and are then transported to an inhibitory cell compartment known as the inhibisome. Therefore, ITAM suppressive signals seem to manipulate lipid raft activating capabilities to encourage receptor inclusion into larger domains, where signalling molecules are deactivated by SHP-1 (Ivashkiv, 2011). In contrast, high avidity binding of FcαRI expressed on the surface of neutrophils was shown to elicit stronger activation signals, as measured by the amount of ERK phosphorylation, and consequently showed greater clearing of cancer cells (Harrison et al., 2022).

While the strength of binding can govern the functional plasticity of the same receptor, the cell type where the receptor is expressed and the adaptors housed in specific cells can determine the resulting cellular outcome. For example, the ITIM and ITAM containing FcRL5 perform varying roles in marginal zone B cells and B1 B cells. Both cell subsets express similar levels of Lyn kinase; however, given the strong signalling of BCR and higher levels of SHP-1 in marginal zone B cells (with SHP-1 levels being twice as high in MZ B-cells), the cross-linking of BCR and FcRL5 in these cells enables FcRL5 to inhibit BCR signalling. On the other hand, B1 B cells, which express a lower level of SHP-1, and have a balanced interaction with Lyn, do not experience the same inhibitory effect on BCR activating signals when FcRL5 and BCR are cross-linked, thus leading to activation of the B1 B-cell (Rostamzadeh et al., 2018). Another example of receptor functional plasticity was observed in the signal lymphocyte activation molecules (SLAM) family proteins. Expressed on macrophages, NK cells, B-cells and T-cells,

SLAM members typically contain multiple ITSM residues in their CYT (Dragovich & Mor, 2018). Although the ITSM amino acid sequence (TxYxx[/I/L/V]); where x is any amino acid) is conserved across different SLAM proteins, the function they mediate, whether inhibitory or activating, depends on the adaptor proteins recruited to the site of engagement. For example, the SLAM-associated protein (SAP)-related molecule, SAP, binds to ITSM via its SH2 domain to competitively prevent the recruitment of phosphatases such as SHP-1 and SHP-2 and simultaneously recruit the activating kinase Fyn. In the case of SLAMF6, which is expressed by CD4+ T cells, the SLAM-SAP-Fyn complex leads to enhanced cellular activation, as seen by increased IL-4 levels (Cannons et al., 2011). When SAP is deficient, in inactivated NK cells as an example, Csk is recruited and binds to the ITSMs of SLAMF6. This results in the dephosphorylation of downstream activating molecules such as Fyn, ultimately suppressing the cytotoxic ability of NK cells.

In summary, various immunoregulatory receptor types play dynamic roles in regulating numerous innate immune responses that aid in eliminating pathogens and maintaining homeostasis. While the presence of immunoreceptor tyrosine-based motifs within immune proteins provides insight into the signalling pathways that regulate these effector responses, the regulatory complexity extends beyond the basic framework of immune receptor activation and inhibition. The context in terms of the type of cell those receptors are expressed in, the adaptors proteins available, and their expression levels are important in determining the overall outcome mediated by immunoregulatory receptors.

2.3 Immunity in Fishes

2.3.1 Overview

Teleosts are a diverse group of fish that make up over 95% of all living fish species and about half of all extant vertebrate species. They are characterized by their homocercal caudal fin, protrusible jaw and complex fin musculature, among a few other unique anatomical traits not found in more primitive fish groups (Helfman et al., 2009). They display remarkable diversity that influences their shape, ecology, and behaviour, among many other biological facets. This vast variability renders them intriguing for exploring numerous biological questions, particularly those related to immunological processes (Jn, 2004). In addition, teleosts act as a bridge between our understanding of innate and adaptive immune responses, given that they represent one of the earliest classes of vertebrates embodying components of both the adaptive and innate immune systems (Whyte, 2007). Therefore, examining immunological processes that helped establish and sustain such a large family will help scientists connect the gaps in the evolution of the immune system, as well as supplement our understanding of current mammalian immune systems.

The fish immune system has physiological similarities to those of higher vertebrates, even though there are certain discrepancies. Unlike land-dwelling vertebrates, fish are free-living organisms from the early embryonic stages of their life and rely heavily on their inherent immune system for survival (Rauta et al., 2012). Given that fish live in an aquatic environment, they are perpetually always exposed to pathogens, hence, nonspecific immunity serves a crucial role as a primary defence system at early stages and throughout the life of fish. Physical barriers such as the skin (including scales and mucus), gills, and the epithelial layer of the GI tract help prevent the invasion of pathogens to internal tissues (Smith et al., 2019). Fish skin contains skin-associated lymphoid tissue (SALT, which comprises various cell types such as secretory cells

(e.g., goblet cells), lymphocytes (i.e., B and T cells), granulocytes, macrophages, and cells similar to Langerhans cells found in mammals (Ángeles Esteban, 2012; Xu et al., 2013). Like mammals, those specialized cells will launch an immediate proinflammatory attack once the skin is breached to eliminate the invading entity.

Humoral factors in teleost fish have been thoroughly studied and include lytic enzymes (i.e., AMPs), proteins of the complement pathways, opsonins, antibodies, and cytokines (Magnadóttir, 2006). These factors, for the most part, are evolutionary conserved and analogous to those of higher vertebrates. For example, chicken (c)-type and goose (g)-type are the two major types of lysozymes in higher vertebrates. They are also found in numerous teleost species, typically in neutrophils, monocytes, and to a lesser degree, in macrophages across various tissues like the liver, kidney, spleen and gills (Saurabh & Sahoo, 2008). When g-type lysozymes from European sea bass (*Dicentrarchus labrax*) were cloned and produced as recombinant proteins, they showed high bacteriolytic potency against several teleost fish pathogens such as *Vibrio anguillarum*, *Aeromonas hydrophila*, and *Micrococcus lysodeikticus* (Buonocore et al., 2014). Like in terrestrial vertebrates, teleost lysozymes break down the peptidoglycan in bacterial cell walls, leading to cell lysis. Another similarity of the teleost innate immune system to mammalian vertebrates lies in the utilization of cytokines to induce anti-viral responses. IFNα and β are cytokines with a generalized antiviral function rooted in their ability to hinder nucleic acid replication within infected cells. IFN plays a crucial role in defending vertebrate host cells against viral infections as these cells can release IFNα/β when they detect viral nucleic acid (Robertsen, 2006). When IFNs bind to their corresponding receptors (i.e., IFN-α/β-receptor, IFNAR), this induces the activation of the Janus kinase-(JAK) and signal transducer and activator of transcription proteins-(STAT) signalling pathway resulting in the expression of

several hundreds of IFN-stimulated genes (ISGs). Some of these genes give rise to antiviral proteins such as protein kinase dsRNA-activated (PKR), and 2,5-oligoadenylate synthetase (OAS) (de Veer et al., 2001; Samuel, 2001). Two types of interferons (IFNα-1 and IFNα-2) have been isolated from the Atlantic salmon and have been studied for their sequence, gene structure, promoters, and their ability to stimulate the antiviral activity of interferon-stimulated genes (ISG) (Kileng et al., 2007; Rokenes et al., 2007). The IFNα-1 from Atlantic salmon triggers the expression of ISGs, and they exhibit similar characteristics to IFNα/β and INFγ in mammals (Rokenes et al., 2007). Additionally, IFNα-1 in Atlantic salmon generates a strong antiviral response against cells infected with pancreatic necrosis virus (IPNV).

While non-specific humoral factors play a major role in teleost immunity, target-specific antibodies (i.e., immunoglobulins) are also found in fish, which are essential for their survival. The main immunoglobulin found in fish is the tetrameric IgM, which contains eight antigen-binding sites (Smith et al., 2019). Certain teleosts have a monomeric form of IgM in their serum, but the conditions that trigger its production remain undetermined. In sharks, monomeric IgM is produced mainly in humoral responses (Voss & Michael Sigel, 1971). Although both the monomeric and tetrameric forms of IgM in rainbow trout share similar binding affinities, the tetrameric version is more efficient in activating the complement system due to a structural variation in the Fc portion of the molecule (Elcombe et al., 1985). IgD was also identified in teleost, specifically in the channel catfish (*Ictalurus punctatus*). IpIgD amino acid sequence resembles that of mammalian IgD, its genomic location falls immediately beneath the IgM gene, and its expression in B cells, all of which are genetic components seen in mammals (Wilson et al., 1997). In addition, IgT/Z is another teleost immunoglobulin protein with a putatively analogous role to mammalian IgAs (Xu et al., 2013; Zhang et al., 2010). For instance, the

quantity of IgT/Z in the serum of rainbow trout is significantly less than IgM, but the IgT/Z to IgM ratio is 63 times greater in the gut compared to the serum (Zhang et al., 2010). Upon intestinal parasitic infection with *Ceratomyxa shasta,* a protozoan parasite with considerable tropism for the gut of salmonids, the count of IgT+ B cells in the gut went up significantly, while the number of IgM+ B cells remained the same; an anti-parasite response that is consistent in higher vertebrates. For example, IgA+ B cells constitute 95% of all B cells that accumulate in the large intestine of mice infected with the parasite *Eimeria falciformis* (Nash & Speer, 1988). Furthermore, IgT+ B cells are also present in the SALT of teleosts, where they release IgT into the skin mucus.

Beyond the physical barriers and humoral factors, teleosts also express a large array of immune receptors. However, our current understanding of their mechanisms is limited. Many of the identified immune receptors in teleost fish belong to the IgSF, with some teleost immune receptor groups sharing common features with the mammalian, bird, and amphibian receptor families (Berstein et al., 1996). One such family is the novel immune-type receptor family (NITRs). Originally discovered in pufferfish, NITRs were also found in teleosts such as channel catfish, rainbow trout, and zebrafish (Yoder et al., 2009; Evenhuis et al., 2007). Typically, they possess one or two extracellular Ig domains that were shown to be related to NK receptors and CD8α/β of the TCR surface proteins complex. Existing in large and conserved gene clusters in the teleost genome, NITRs have been proposed as functional equivalents of mammalian NK receptors as they have been reported to recognize allogeneic targets (Yoder et al., 2001; Desai et al., 2008). Additionally, they feature TM segments and CYT regions of varying lengths with different configurations of signalling motifs (i.e., ITIM-like, ITAM-like, or both). Most NITRs are considered to be inhibitory due to their ITIM-like-containing CYTs, however, a relatively

minor subset of NITRs is thought to be activating receptors, owing to the absence of ITIMs and the presence of a positively charged residue in their TM regions. When transfected and expressed in HEK 293T cells, Nitr9L was shown to preferentially co-aggregate with a zebrafish ortholog of DAP12, inducing cell activation via a PI3K-dependant pathway (Wei et al., 2007).

A possible teleost homologue of vertebrate FcRs was identified during an examination of existing genome databases for the channel catfish. Termed IpFcRI, this receptor contained extracellular 3 Ig-like domains, but it lacked a TM and CYT, suggesting that this protein might be secreted (Stafford et al., 2006). Initial protein modelling of IpFcRI indicated that the two distal Ig-like domains (D1 & D2) share similarities with other FcRs and incorporate a hypothetical Ig binding site. Transcriptional analysis of peripheral blood leukocytes (PBL) showed that IpFcRI mRNA is expressed in granulocytes and lymphocytes, however, it was also found that the expression of IpFcRI can vary by tissue and cell type (Stafford et al., 2006). When a recombinant IpFcRI was expressed in an insect cell-line expression system, IpFcRI was confirmed to be a secreted protein and was also identified in catfish plasma using polyclonal antibodies. Despite being secreted, co-immunoprecipitation (co-IP) experiments showed that IpFcRI binds to serum-derived fish IgM, indicating it is a teleost FcμR (Stafford et al., 2006).

Overall, effector responses of the innate immune system are well conserved between fish and mammals, but the immunoregulatory receptor-types and their related signaling networks are not as extensively researched in fish. Functionally, teleost macrophages and B cells have been shown to perform phagocytosis (Esteban et al., 2015), mast cells and neutrophils trigger degranulation (Sfacteria et al., 2015; Havixbeck & Barreda, 2015), and fish neutrophils/NK cells lead to cell-mediated cytotoxicity (Kurata et al., 1995; Yoshida et al., 1995). Despite these responses in fish mirroring those in mammals, considerable gaps in understanding persist about

how fish immune cell effector functions are regulated. Of late, numerous fish immunoregulatory families have been uncovered thanks to molecular cloning and the availability of teleost genomes. The investigation of these families is now offering comparative immunologists new insights into how fish immunoregulatory networks are initiated, sustained, and/or inhibited.

2.3.2 Common fish Models used to Study Immunity

2.3.2.1 Channel catfish

The channel catfish (*Ictalurus punctatus*) has proven to be a valuable comparative model for studying cellular immunity in fish. Partly, this is due to the unique ability of catfish peripheral blood leukocytes (PBLs) to survive in culture for extended periods without needing transformation, which has facilitated the development of several clonal lymphoid and myeloid catfish cell lines, opening an avenue for cell-based research (Clem et al., 1996). Additionally, the catfish genome has been extensively analyzed over the past three decades. Significant advancements have been made, including the development of large-scale molecular markers, the construction of high-density genetic maps, Bacterial Artificial Chromosome (BAC)-based physical maps, and the integration of these genetic maps with physical maps (Yáñez et al., 2016). Extensive expressed sequence tags (ESTs) libraries have also been produced from both channel catfish and blue catfish, which later helped in the discovery and characterization of several immunoregulatory receptors.

Clonal channel catfish lymphoid cell lines (e.g. TS32.15, 3B11) and mixed lymphocyte cultures (MLCs) have been beneficial for studying immune responses at both the cellular and molecular levels (Majji et al., 2009). Various techniques such as reverse transcription polymerase chain reaction (RT-PCR), along with Northern and Southern blotting, have been used for the biological and phenotypical characterization of these MLCs, and clonal catfish cell

lines. Microarray analysis, a tool used to detect the expression of thousands of genes at the same time, has been further used to extend the molecular understanding of these cultures. For instance, the clonal B cell (3B11), the macrophage cell line (42TA), the cytotoxic T cell lines (TS32.15 and TS32.17), and polyclonal MLCs were analyzed using a cDNA array, which comprised roughly 2500 probes derived from EST libraries made from the 42TA macrophage cell line, MLCs, and young catfish fry aged between 5-14 days. The analysis revealed that each cell line expressed a unique RNA profile encompassing a wide range of immune-related genes.

Collectively, the establishment of catfish clonal cell lines and the subsequent characterization of immune-related genes have opened the gates for catfish to be an excellent model for studying aspects of innate immunology. Immune genes such as those encoding PRRs, AMPs, complements, lectins, and cytokines have been extensively studied in the catfish model, and insights drawn from them complemented our understanding of vertebrate immunity (Zhou et al., 2012; Zhang et al., 2012; Pridgeon et al., 2012; Rajendran et al., 2012; Rajendran et al., 2012). For instance, a total of 5 functional TLRs (TLR2, TLR3, TLR5, TLR20 and TLR21) have been characterized in catfish. Expression analysis upon challenge with *Edwardsiella ictaluri* showed no direct correlation between those PRRs and susceptibility to enteric septicemia of catfish (ESC) (Gao et al., 2012). However, it was later proposed that TLR3 was associated with a broader immune response and TLR5 aided in the accumulation of macrophages during an *E. ictaluri* infection (Bilodeau & Waldbieser, 2005; Bilodeau-Bourgeois et al., 2008). In contrast to mammalian TLR5, catfish possess two isoforms of TLR5: a membrane-bound variant (TLR5M) and a soluble variant (TLR5S). The TLR5M is analogous to mammalian TLR5, however, the TLR5S, which lacks a transmembrane domain and a TIR domain, doesn't exist in mammals. Upon co-transfecting and co-expressing both receptors in a human embryonic kidney cell line

(HEK-293T), TLR5S was shown to bind to TLR5M and inhibit its further expression while inducing the expression of downstream proinflammatory factors such as TNFα and IL-8 (Ouyang et al., 2022). While considered the most extensively studied PRRs, insights from catfish TLR5M-TLR5S expanded our understanding of how these receptors can be regulated.

Regarding humoral factors examined in channel catfish, infection with *E. ictaluri* was correlated with increased production of two AMPs, hepcidin (LEAP-1) and liver-expressed antimicrobial peptide 2 (LEAP-2), in most catfish tissues (Bao et al., 2006; Gao et al., 2012). Both the amino acid sequences and gene organization of hepcidin and LEAP-2 showed a significant level of conservation with those found in other species, including pigs, rhesus monkeys and cows. However, the channel catfish hepcidin gene showed a broader tissue expression than its homologs in mammals, which are primarily expressed in the liver (Douglas et al., 2003; Zhou et al., 2011). Its expression was also noted early during embryonic and larval development. Six days-post hatching, the mature mRNA of LEAP-2 in Channel catfish was consistently detected at stable levels, implying a potential role as self-generated AMPs in safeguarding embryos against pathogens.

2.3.2.2 Zebrafish

Zebrafish (*Danio rerio*) has been utilized in biomedical research to investigate vertebrate development, hematopoiesis, and, more recently, immunological processes (Berton et al., 2005). Zebrafish larvae rely solely on their innate immune responses for survival, as their adaptive immune system only becomes morphologically and functionally mature 4–6 weeks after fertilization. This distinct timing offers an opportune system to independently study the vertebrate innate immune response *in vivo*. The transparency of zebrafish during early life stages allows for beneficial real-time visualization experiments. Adult zebrafish, which possess both

innate and adaptive immune systems, also provide advantages over other vertebrate infection models due to their small size, relatively quick life cycle, ease of breeding, and an expanding array of molecular tools for studying infectious diseases.

Several immune genes and proteins in zebrafish share homology with those found in mammals, including ISGs (such as *psmb9*, immunity-related GTPases (IRGs), and IFNs) which show increased expression in the presence of IFN-γ1 and IFN-γ2 (Sieger et al., 2009). Further investigation revealed that IFN-γ signalling plays a pivotal role in the resistance against bacterial infections in zebrafish embryos. This was indicated when a reduction in survival rates was observed in IFN-γ knockout embryos during infections with *E. coli* and *Yersinia ruckeri*. In addition to immune genes, zebrafish contain receptor-types related to receptors found in higher vertebrates as well as novel fish-specific receptor families such as NITRs (Trede et al., 2004); Yoder et al., 2009). Zebrafish possess roughly 24 variants of TLRs, with ten of them sharing orthologous characteristics with specific human TLRs, including TLRs 2, 3, 4, 5, 7, 8 and 9. Zebrafish TLR22 shows a significant resemblance to the toll 9 gene found in *Drosophila melanogaster* and belongs to a fish-specific subfamily. TLR21, on the other hand, is only detected in fish, amphibians, and birds. Among these identified receptors in zebrafish, some are speculated to be potential spliced variants (for example, TLR 4.1a and 4.1b), while others could be the product of gene duplications (like TLR4.1 and 4.2). Furthermore, downstream signalling components following TLR activation have been identified, with molecules such as MYD88, the TIR domain-containing adaptor inducing interferon-β (TRIF), IRF3, and IRF7 present in zebrafish.

In addition to immune genes and receptor-types, zebrafish embryos develop various immune cells that are analogous to those found in mammals, such as lymphocytes,

monocytes/macrophages, and neutrophils (Mathias et al., 2006). This allows scientists to study the activity of immune cells in response to infection and draw insights into how they might behave in mammals. For instance, the primary method for resolving neutrophil-driven inflammation involves the controlled apoptosis of neutrophils in damaged tissues. Yet, observations of GFP-tagged neutrophils in transgenic zebrafish reveal that inflammation resolution can also be achieved through the reverse migration of neutrophils from the injured tissue to the bloodstream. Overall, zebrafish was shown to be an excellent model to study aspects of the vertebrate innate immune system thanks to features such as rapid growth, range of immune cells, and presence of immune genes like that of mammals.

2.3.2.3 Goldfish

The use of unconventional model organisms is seeing a marked increase across various research disciplines, thereby significantly enhancing our understanding of life sciences. For example, *Carassius auratus*, commonly known as the goldfish, is extensively utilized in studies focused on comparative and evolutionary endocrinology, neurobiology, immunology, and mechanisms of cell development governing all these physiological systems (Filice et al., 2021). Additionally, goldfish are used in translational research aimed at uncovering mechanisms that could be beneficial in a practical biomedical context (Ferreira et al., 2014; Imbrogno et al., 2014; Jacquin et al, 2019)

For instance, colony-stimulating factor-1 (CSF-1) is the key growth factor responsible for managing the survival, proliferation, and differentiation of mononuclear phagocytes and their precursors (Mroczko et al., 2002). Besides this primary role, CSF-1 has been shown to contribute significantly to processes such as bone metabolism, the formation of atherosclerosis, inflammation, pregnancy, and the development of the female reproductive tract prior to

implantation (Pixley & Stanley, 2004). Goldfish CSF-1 is the first CSF-1 to be identified and functionally characterized in non-mammalian organisms (Barreda et al., 2004, Hanington et al., 2009). Despite being significantly smaller than its mammalian counterpart, goldfish CSF-1 possesses the necessary amino acids in the correct biochemical positions to form a functioning peptide. Goldfish CSF-1 functions in a similar manner to mammalian CSF-1, as it prompts the differentiation and proliferation of fish mononuclear cells by interacting with its specific receptor, the CSF-1R. Recombinant goldfish CSF-1 (rCSF-1) was introduced to sorted goldfish progenitor cells, monocytes, and macrophages two days post-cultivation at concentrations ranging from 1 to 50 ng/ml. The growth of these cells was monitored daily using flow cytometry. Cells treated with rCSF-1 were then compared to cells treated with cell culture media (CCM; positive control), elution buffer, and cultures without any treatment. Progenitor cells and macrophages did not show any changes upon the addition of rCSF-1. However, sorted monocytes developed into mature macrophages much earlier when treated with rCSF-1 compared to those incubated with CCM (Hanington et al., 2007). When a soluble variant of the goldfish CSF-1 receptor (sCSF-1R) was pre-incubated with rCSF-1, the proliferation of sorted monocytes was diminished, suggesting a unique regulatory mechanism of goldfish CSF-1, unlike that seen in mammals. Specifically, studies of CSF-1 in mice models have shown that CSF-1 is quickly cleared from the bloodstream by sinusoidal macrophages, primarily by liver-specific Kupffer cells. CSF-1 attaches to CSF-1R, and the whole complex gets internalized and subsequently degraded (Bartocci et al., 1987).

Another area in which a wealth of knowledge was obtained from using goldfish as a model organism is the examination of myelopoiesis, which is a collective term that describes the process by which hematopoietic stem cells (HSCs) or hematopoietic progenitor cells (HPCs),

which are limited in number, develop into myeloid cells such as macrophages, neutrophils or granulocytes, thrombocytes, and erythrocytes. The application of goldfish as a model for studying myelopoiesis was first put into practice in the 1990s with the introduction of a unique *in vitro*-based primary kidney macrophage (PKM) culture system (Neumann et al., 1998; Neumann et al., 2000). The dominant pathway of macrophage development observed in these PKM cultures was the classic approach, where progenitor cells evolve into monocytes and, subsequently, macrophages (Barreda et al., 2000). This development of macrophages in goldfish PKM cultures was linked to the generation of innate growth factors by the supposed progenitor cell population and mature macrophages (Neumann et al., 2000). Interestingly, an alternate macrophage development pathway was occasionally noted, where progenitor cells progressed to macrophages without a significant monocyte stage (Barreda et al., 2000). The exact conditions prompting the shift from the classic to the alternative macrophage development pathway remain unknown. Yet, it could be speculated that this alternate pathway may serve as a swift route to generate mature macrophages during emergencies *in vivo*. Uniquely among teleost models, the goldfish PKM system allows a thorough examination of interactions between concurrently developing macrophage subsets *in vitro*.

Following the implementation of the PKM culture system, large quantities of highly pure populations (>92%) of goldfish primary kidney neutrophils (PKNs) were identified and isolated (Katzenback & Belosevic, 2009). Functional studies showed that goldfish PKNs had similar capabilities to mammalian neutrophils, including chemotaxis, degranulation, production of reactive oxygen intermediates, and phagocytosis in response to mitogens or fish pathogens such as *Aeromonas salmonicida* or *Mycobacterium marinum* (Havixbeck et al., 2015). Molecular characterization of goldfish neutrophils revealed the expression of transcripts for

myeloperoxidase, granulocyte colony-stimulating factor receptor (G-CSFR), tyrosine kinase receptors (kit), prominin, myeloid transcription factors, and pro-inflammatory cytokines such as IL-8, IFN-γ, TNF-α, IL-12 and inducible nitric oxide synthase (iNOS) (Havixbeck et al., 2015; Ortega et al., 2013). Overall, goldfish PKNs seem to mirror mammalian neutrophils and could be very useful for investigating how goldfish neutrophils respond to pathogens and environmental contaminants *in vitro*.

TNF-Receptor 1 and TNF-Receptor 2 (TNF-R1 and TNF-R2, respectively) were also the focus of extensive studies in the goldfish system (Grayfer & Belosevic, 2009). The expression levels of these receptors in different tissues and immune cell types were examined using qPCR, and results showed that TNF-R2 was expressed significantly more than TNF-R1 across all tissues. The highest expression of both receptors was observed in monocytes, while the lowest expression of TNF-R1 was found in mature macrophages and that of TNF-R2 in peripheral blood leukocytes. In mammals, the TNF-α ligand binds to TNF-R1 and TNF-R2, which in turn trimerize upon engagement. Binding experiments suggest that both recombinant goldfish TNF-α-1 and TNF-α-2, and recombinant extracellular domains of goldfish TNF-R1 and TNF-R2, primarily form dimers rather than trimers (Grayfer & Belosevic, 2009). The potential functional significance of these ligand-receptor interactions is substantiated by findings showing that goldfish TNF-R1 and TNF-R2 can suppress the macrophage reactive oxygen species (ROS) responses primed by regulatory TNF-α-1 or regulatory TNF-α-2. This marked the first report of a TNF receptor-ligand interaction in a teleost species and the first documentation of dimerization of a fish TNF-R1 or TNF-R2 (Li et al., 2020)

When it comes to receptors of the IgSF, an examination of the goldfish genome revealed a total of 12 NITR members clustered on chromosome 7 (Wang et al., 2020) Moreover,

sequences of receptors with Ig-like domains that are closely related to Ig-like domains of various mammalian classical FcRs were found in the goldfish genome, however, they do not appear to be members of an extended gene family. Apart from expression analysis upon pathogen infection (Tu et al., 2019; Das et al., 2021), little is known about how goldfish receptors can modulate immune effector functions, which signalling molecules are involved, and what conditions govern the functional plasticity of such a diverse collection of immune receptor types.

Overall, the recently published goldfish genome (Chen et al., 2019), alongside the RNA transcriptome (generated in our lab) has further advanced the utility of the goldfish research model to study immune genes and their correspondent immune proteins. Having well-established PKMs and PKNs goldfish cultures (Katzenback et al., 2016), on top of the recently established techniques for investigating the functional capacities of immunoregulatory proteins (described below), presents an opportunity to explore the immunoregulatory roles of goldfish receptor types, and their variants in either goldfish myeloid cells or using mammalian cell lines. Therefore, the goldfish model system serves as an important platform for continued exploration of immunoregulatory events in teleosts.

2.4 Teleost Leukocyte immune-type receptors (LITRs)

2.4.1 Overview

Leukocyte immune-type receptors (LITRs) are a large family of teleost immunoregulatory receptor-types belonging to the immunoglobulin superfamily (IgSF). These immune genes are phylogenetically and syntenically related to Fc receptor-like protein genes (FcRLs) present in other vertebrates, including amphibians, birds, mice, and man. *In vitro*-based functional analyses of LITRs have shown that LITRs have diverse immunoregulatory potentials, including the activation and inhibition of effector responses such as cell-mediated cytotoxicity,

degranulation, cytokine secretion, and phagocytosis; mainly due to their unique tyrosine-containing CYT regions (Montgomery et al., 2012; Cortes et al., 2014; Lillico et al., 2015). LITRs were also shown to exhibit functional plasticity when expressed in different cell lines, as well as the ability to modulate the phagocytic ability of AD293 cells when cross-linked using antibodies (Fei et al., 2020). In the following sections, I will review the knowledge accumulated throughout the years of studying LITRs since their original description in 2006.

2.4.2 Genomic Organizations, Phylogeny and Syntney of Teleost LITRs

In vertebrates, gene families of related immunoregulatory receptor-types are usually found in large genomic clusters characterized by unique genomic landmarks, such as physical linkage to other genes, the chromosome they are found in, and the gene content/order/transcription direction (Ohta et al., 2019; Trowsdale et al., 2001) Often, the organization of these gene clusters is conserved across vertebrates (Akula et al., 2014; Davis et al., 2002; Barten et al., 2001). For example, *fcrs* and *fcrls* are located in large genomic clusters on human chromosome 1q21-23 (Guselnikov et al., 2002). The distal Ig-like domains (D1, D2, D3) of *fcrl* family members are distantly related to the Ig-like domains of the classical *fcrs* (CD64, CD42, and CD16). These are genomically linked to genes from the CD2 superfamily (like *slam* family genes) and a host of other genes, including *dusp12*, *etv3*, *cd5l* and *nges* (Davis, 2007). In a similar fashion, the human leukocyte receptor complex (LRC), found on chromosome 19q12.3, includes genes such as *lilrs* and *kirs*. They are clustered together with various phylogenetically unrelated genes such as tweety family member 1 (*ttyh1*), leukocyte receptor cluster member 8 (*leng8*), and leukocyte receptor cluster member 9 (*leng9*) (Sambrook et al., 2006). In mice, the paired immunoglobulin-like receptors (*pirs*) are genomically placed within relatives of human *ttyh1, leng8, leng9*, and several other genes from the human LRC region

(Martin et al., 2002). In other vertebrates, relatives of human *lilrs* and mouse *pirs* have been identified in an LRC-like area in the tropical clawed frog (*Silurana tropicalis* immunoglobulin-like receptor (*silrs*)) positioned near several relatives of mammalian genes *ttyh1, leng8, and leng9* (Guselnikov et al., 2008). Thus, akin to *fcrs* and *fcrls*, human *lilrs* and their mouse and amphibian counterparts belong to a genomic complex with conserved synteny.

When catfish *litrs* were first identified and characterized (Stafford et al., 2006), phylogenetic analysis of their expressed sequences suggested they were related to mammalian LRC and FcR members. Specifically, the proximal D3 and D4 Ig-like domains of *litr1* and *litr3* were shown to be phylogenetically related to the Ig-like domains of mammalian *kirs*, *lilrs* and the natural killer cell p46-related protein (NKp46). In contrast, the distal D1 and D2 Ig-like domains of catfish *litr1*, *litr2*, and *litr3* grouped more closely with the Ig-like domains of mammalian *fcrs* and *fcrls*. Therefore, IpLITRs showcased a unique "chimeric" composition of Ig-like domains reminiscent of multiple mammalian immunoregulatory receptor families with diverse functions. Similarly, goldfish *litrs* (CaLITRs) and zebrafish *litrs* (DrLITRs) have phylogenetic traits that resemble those of IpLITRs, suggesting a level of conservation in the genomes of teleost. Detailed phylogenetic and syntenic analysis of teleost *litrs* are found in (Wang et al., 2021), and our lab used bioinformatics tools such as Ensembl genome databases, BLAST, and MEGA7 to investigate the genomic organization and potential presence of *litrs* orthologs in channel catfish, zebrafish, goldfish, and pufferfish fugu. Overall, teleost *litrs* are tightly linked (frequently less than 1 million base pairs apart) and exist on multiple chromosomes in each fish species examined. Even though they are situated on various chromosomes, the bulk of the *litr* genes in each teleost is found on a single chromosome, such as chromosome 7 in goldfish, zebrafish, and catfish. This suggests that *litrs* were probably originally located on one

chromosome in the ancestral teleost, and later genomic events like teleost-specific genome duplication (TGD), gene conversion, and chromosomal translocation moved some *litrs* to different chromosomes. Moreover, the allotetraploidization event is likely the reason why *litrs* are spread across seven different chromosomes in goldfish (Yuan et al., 2010; David, 2003).

Next, our lab looked at potential syntenic relationships between the *litr* clusters. Generally, conserved synteny refers to the preserved genomic organization of orthologous genes in a specific chromosomal region across two different taxa (Catchen et al., 2009). Our findings reveal that the zebrafish *litrs* cluster on Chr 7 is associated (less than 3 million base pairs apart) with several neighbouring 5' genes, including *myadm, vangl2, arhgef11*, and the cluster of *slam* family gene (Wang et al., 2021). Similarly, a goldfish *litr* cluster on Chr 7 is also associated with similar genes, including three *SLAM* family genes, three *vangl2s*, and two *myadms*. This evidence strongly indicates that the zebrafish *litr* cluster on Chr 7 and the goldfish *litr* cluster on Chr 7 represent conserved syntenic chromosomal regions. Furthermore, two distinct *litr* clusters found on channel catfish Chr 7 are both connected to five *slam* family genes, *vangl2*, *arhgef11*, and two *myadm* genes. Similarly, a single *litr* cluster found on fugu Chr 8 is connected to a *SLAM* family gene, *vangl2, arhgef11*, and two *myadm* genes. Taken together, these results suggest that different teleost genomes have *litr* clusters displaying conserved synteny. Worth noting is the presence of other *litr* clusters and individual *litr* genes that do not appear to be associated with any of the neighbouring genes (*SLAM* family, *arhgef11, vangl2, and myadm*) found in the conserved *litr* syntenic regions of each examined teleost. These *litrs* also do not exhibit physical linkage relationships with any other genes based on the study above.

Having established the conserved syntenic *litr* genomic regions in teleost fishes, our lab subsequently examined whether *arhgef11, vangl2, myadm, and cd2* (*slam* gene family) are also

connected to the *fcrl* genomic regions in the tropical clawed frog (*Xenopus tropicalis*), chicken (*Gallus gallus*), mouse (*Mus musculus*), and human (*Homo sapiens*). It was found that genes corresponding to teleost *cd2, vangl2, and arhgef11* are also linked (less than 1 million base pairs apart) with *fcrl* gene clusters in frogs, mice, and humans, with the unique exception of the chicken genome (Wang et al., 2021). The *fcrl* gene repertoire in chickens is notably diminished, which is likely attributable to the significant decrease in protein-coding genes in the chicken genome compared to mammals (Hughes & Friedman, 2008). Subsequently, phylogenetic analyses were carried out to compare the Ig-like domains of goldfish *litrs* with those of *fcrls* identified in the tropical clawed frog, chicken, mouse, and human. Two distinct types of goldfish *litrs* Ig-like domains (D1 and D2) show sequence similarity with the distal Ig-like domains (D1, D2, and D3) present in the tropical clawed frog, chicken, mouse, and human *fcrls* investigated. To reinforce the evolutionary relationship between the Ig-like domains of *litrs* and those of mammalian *fcrls*, additional phylogenetic analyses were performed to determine if the complete extracellular domains (as opposed to individual Ig-like domains) of representative *litrs* also group with the full-length extracellular domains of representative mammalian *fcrls* (Wang et al., 2021). Overall, our findings support the individual Ig-like domain phylogenetic trees and clearly demonstrate that the two representative goldfish *litrs* (CaLITR5 and IpLITR3) preferentially group with higher vertebrates *fcrls*, but not with members of the mammalian LRC (such as *kirs*, and *lilrs*)

Overall, the conserved synteny and the phylogenetic analyses described above indicate that teleost *litrs* are likely to be homologs of *fcrls* found in amphibians and terrestrial animals including mammals. They also show that *litrs* are only found teleost fish, and not in earlier basal ray-finned fish (spotted gar); suggesting that *litrs* are teleost-specific. These findings pave the

way for a more in-depth exploration of potential functional similarities between teleost LITRs and FcRLs.

2.4.3 Examination of Stimulatory LITR-Mediated Responses

Early functional studies focused on an isoform of the previously mentioned IpLITR2, known as IpLITR 2.6b, which contains a short CYT region with a positively charged lysine residue in its TM segment. Like classical activating receptors of the IgSF, it was predicted that this receptor would associate with tyrosine-containing adaptor proteins upon surface engagement, leading to surface expression and activation (Mewes et al., 2009). This hypothesis was tested using HEK-293T cells co-transfected with a hemagglutinin (HA)-tagged N terminus of IpLITR 2.6b, along with FLAG-tagged, negatively charged catfish adaptor proteins (i.e., IpFcRγ, IpFcRγ-L, IpCD3ζ-L, or IpDAP12), each containing a single ITAM motif within their CYT regions. Flow cytometry and western blotting studies showed that IpLITR 2.6b associates with IpFcRγ, IpFcRγ-L, and IpCD3ζ-L, and the association with IpFcRγ and IpFcRγ-L (but not IpCD3ζ-L) led to an enhanced cell surface expression of IpLITR 2.6b (Mewes et al., 2009). It was also observed that DAP12, an adaptor commonly associated with other members of the IgSF and possessing lysine residues in their TM segments (Hamerman et al., 2009; Feng et al., 2006), did not associate with IpLITR 2.6b. Overall, these biochemical analyses validated the association of a putative stimulatory IpLITR-type with teleost-specific ITAM-containing adaptors, revealing intriguing new insights about the specificity of these interactions.

To evaluate the signalling potential of the IpLITR 2.6b-IpFcRγ-L complex, a chimeric receptor was created. This involved fusing the extracellular domains of IpLITR 2.6b with the transmembrane and ITAM-containing CYT region of IpFcRγ-L (Cortes et al., 2012). The chimera IpLITR 2.6b/IpFcRγ-L (referred to simply as IpLITR 2.6b below) was tagged with an

N-terminal HA tag, allowing it to be stimulated using anti-HA specific monoclonal antibodies (mAbs), followed by stable transfection, and expression in the rat basophilic leukocyte cell line (RBL-2H3). Upon stimulation with anti-HA mAbs, IpLITR 2.6b induced degranulation by RBL-2H3 cells. However, mutating the functional CYT tyrosine within ITAM to a non-functional phenylamine nullified this response, verifying, as expected, that these responses are ITAM-dependent (Cortes et al., 2012). IpLITR 2.6b activation also led to the phosphorylation of the extracellular signal-regulated kinase (ERK) 1/2 protein as well as protein kinase B (PKB/Akt), which was absent when the receptor with the mutated ITAM was engaged. The use of pharmaceutical inhibitors that target critical intracellular signalling molecules resulted in substantial reductions in the degranulation induced by IpLITR 2.6b, due to the disruption of Src family kinases (SFKs), mitogen-activated protein kinases 1 and 2 (MEK1 and MEK2), PI3K, and PKC. Conversely, inhibitors of the p38 subunit in MAPK and c-Jun N-terminal kinases (JNKs) pathways did not significantly inhibit degranulation. Additionally, IpLITR 2.6b-expressing cells initiated ITAM-dependent phagocytosis when exposed to 4.5 μm polystyrene beads opsonized with anti-HA. Collectively, these data were the first evidence showcasing the functional signalling events mediated by IpLITR 2.6b, suggesting that this stimulating type of IpLITR employs traditional kinase-dependent signalling pathways, as well as an ITAM-dependent phagocytosis mechanism.

The functional potential of IpLITR 2.6b was further explored through a MAPK signalling array and cytokine secretion profiling. Activation of IpLITR 2.6b led to increased phosphorylation of various intracellular signalling molecules, such as ERK1/2, GSK-3α/β, GSK-3β, RSK1, CREB, JNK (pan), MEK6, MSK2, p38δ, and Akt2 (Cortes et al., 2012), providing a more comprehensive perspective on the intracellular signalling components phosphorylated

following the activation of IpLITR 2.6b. Furthermore, these studies showed that IpLITR 2.6b-activated-cells secreted IL-3, IL-4, IL-6, and TNF-α at levels similar to those observed after cell activation with phorbol myristate acetate (PMA), calcium (Ca2+) ionophore, or endogenous FcεRI stimulation (Cortes et al., 2014) This indicated that IpLITR 2.6b/IpFcRγ-L-mediated signalling events simultaneously induced cellular degranulation and cytokine secretion over a similar timeframe.

In mammals, activating receptors like FcRs also form associations with the ITAM-containing adaptor FcRγ-chain (Holowka et al., 2007; Ghazizadeh et al., 1995). The phosphorylation of FcRγ-chain ITAMs by SFKs results in the recruitment of Syk. Syk then acts as the primary effector of FcR-mediated phagocytic signalling by directly binding to and/or phosphorylating other downstream intracellular molecules. Such molecules include PI3K, Vav, Rho family GTPases (e.g., Ras-related C3 botulinum toxin substrate; Rac1 and cell division control protein 42; Cdc42), and their activation results in the triggering of the F-actin polymerization machinery to induce phagocytic cup formation during the early stages of phagocytosis (Park & Cox, 2009; Rosales & Uribe-Querol, 2017). To compare the role that specific signalling molecules play in FcRγ-mediated phagocytosis to that of the one previously observed by IpLITR2.6b, another pharmacological assay was conducted using a flow cytometric-based phagocytosis assay (Lillico et al., 2015). Overall, the inhibition of SFKs, Syk, PI3Ks, PKB/Akt, Cdc42, Rac1/2/3, MEK1/2, phosphoinositide-dependent kinase 1 (PDK1), PKC, and F-actin polymerization abrogated the phagocytic response. Confocal microscopy was used to visualize IpLITR 2.6b-mediated phagocytosis, which further validated the receptor's ability to initiate an ITAM-dependent phagocytic phenotype, similar to what is observed in mammals (Zhang et al., 2010). These findings suggest that IpLITR 2.6b controls ITAM-dependent

phagocytic responses by utilizing signalling molecules like those in the known mammalian FcR-mediated phagocytic pathway.

2.4.4 Examination of Inhibitory LITR-Mediated Responses

As discussed in section 2.2.4, inhibitory receptors regulate the activation potential for immune cells and reduce the effector functions induced by stimulatory receptors. The inhibitory effects largely rely on the phosphorylation of ITIMs, their recruitment, binding, and activation of SH2 domain-containing cytoplasmic phosphatases (such as SHP-1 and SHP-2), which subsequently dephosphorylate downstream signalling molecules leading to the inhibition of the immune cell (Burshtyn et al., 1999). To explore the inhibitory potential of certain putative inhibitory IpLITR-types, receptor chimeras were synthesized by merging the cytoplasmic regions of IpLITR 1.2a or IpLITR 1.1b with the transmembrane and extracellular domain of the human NK receptor KIR2DL3 (Montgomery et al., 2009). As the natural ligand for LITRs remains unknown, using the extracellular domain of KIR2DL3 and its ligand (i.e., HLA-Cw3) became a useful approach to artificially stimulate IpLITR-mediated CYT signalling events. IpLITR 1.2a contains an ITIM and an ITSM, whereas IpLITR 1.1b contains two ITIMs (Y477 and Y499) and an ITSM (Y503) within the membrane distal region of its cytoplasmic tail, as well as a membrane proximal region that doesn't contain recognizable tyrosine-based signalling motifs, although it does have three tyrosine residues (Y433, Y453 and Y463) (Stafford et al., 2006; Montgomery et al., 2009). Upon transiently expressing these chimeras in HEK-293T cells, both SHP-1 and SHP-2 were shown to be recruited to each receptor chimera following the phosphorylation of tyrosines within their ITIMs, yet the membrane-proximal region of IpLITR 1.1b didn't recruit any phosphatases (Montgomery et al., 2009).

Expanding on the recruitment of inhibitory phosphatases, our lab was interested to see if those chimeric IpLITR constructs were able to inhibit well-studied immune responses. Using a vaccinia virus expression system, IpLITR 1.2a and IpLITR 1.1b chimeras were expressed in mouse spleen-derived cytotoxic lymphocytes (Montgomery et al., 2012). Then using HLA-Cw3-expressing B cell targets in a cytolysis assay, our lab investigated the inhibitory capacity of those chimeras, with the goal of resolving the underlying and specific signalling pathways involved. The potential signalling role of the membrane-proximal region of IpLITR 1.1b, which does not contain any ITIMs, was also investigated. These experiments showed that the CYT region of IpLITR 1.2a inhibits NK cell killing of B cell targets via a SHP-dependent mechanism. However, while the CYT region of IpLITR 1.1b was also capable of inhibiting cell killing, the receptor's ability to induce inhibition was not completely dependent on the ITIM-mediated recruitment of SHP-1 (Montgomery et al., 2012). This conclusion was drawn from subsequent experiments where the inhibitory role of the IpLITR 1.1b specific CYT regions was examined. To do this, chimeric constructs containing either the membrane-proximal (IpLITR 1.1b $^{\text{PROXIMAL CYT}}$) or distal (IpLITR 1.1b $^{\text{DISTAL CYT}}$) regions were created and transfected into the same cell line as above. Carrying out the same cytolysis experiments, it was revealed that IpLITR 1.1b $^{\text{DISTAL CYT}}$ and, unexpectedly, IpLITR 1.1b $^{\text{PROXIMAL CYT}}$ both independently inhibited cellular killing responses. The suppression of cellular killing by IpLITR 1.1b $^{\text{DISTAL CYT}}$ was reliant on SHP-1 recruitment, while 1.1b $^{\text{PROXIMAL CYT}}$ was SHP-independent. It was then determined that 1.1b $^{\text{PROXIMAL CYT}}$ induced inhibition through a series of amino acids resembling the Csk binding motif consensus sequence, which was confirmed through co-immunoprecipitation. In mammals, Csk is a kinase that restrains cellular signalling via phosphorylation of the negative regulatory site on SFKs, thus inhibiting its catalytic activity (Okada, 2012). In summary, the inhibitory

IpLITR-types 1.2a and 1.1b limit lymphocyte-mediated cytotoxicity through a SHP-dependent mechanism. However, IpLITR 1.1b also employs a SHP-independent mode of inhibition through Csk binding to its membrane proximal region, suggesting that IpLITRs possess flexible abilities to inhibit immune cell effector responses.

2.4.5 Functional Plasticity of IpLITRs

As with the previously mentioned stimulatory receptor IpLITR 2.6b, the inhibitory receptor IpLITR 1.1b was also transfected and expressed in RBL-2H3 cells to further examine its immunoregulatory potentials (Cortes et al., 2014). Interestingly, the engagement of IpLITR 1.1b with anti-HA opsonized 4.5 μm polystyrene bead targets led to the phosphorylation of signalling molecules, including Erk1/2 and Akt, and initiated a phagocytosis response that was independent of the recruitment of ITAM-associated adaptor molecules. Unlike IpLITR 2.6b, IpLITR 1.1b did not trigger cytokine release, and the kinetics of IpLITR 1.1b-driven phosphorylation of Erk1/2 and Akt markedly differed from the ITAM-mediated responses. Additionally, the phagocytic response triggered by IpLITR 1.1b was not affected by the application of the extracellular calcium chelator EDTA, in contrast to the reduction seen with the addition of EDTA to the IpLITR 2.6b-expressing cells. This further made the IpLITR 1.1b signalling mechanism distinct from that of IpLITR 2.6b. To investigate whether the IpLITR 1.1b-mediated phagocytic response is CYT-region mediated, a CYT-region-truncated IpLITR 1.1b construct was synthesized, transfected, and stimulated. Despite still being expressed on the cell surface, the phagocytic response was not observed. This implies that the response was explicitly induced by the IpLITR 1.1b CYT region and not by an unanticipated association with another immunoregulatory protein(s) (Cortes et al., 2014). Administering the pharmacological inhibitor of F-actin

polymerization, Cytochalasin D, did inhibit the IpLITR1.1b-mediated phagocytic response, indicating that as expected this this response required F-actin polymerization.

IpLITR 1.1b was not the only receptor with predicted and observed inhibitory potential to induce a phagocytic response in RBL-2H3 cells. Using the same system described above, IpLITR 1.2a was also found to induce phagocytosis. Overall, the complexity in which this receptor family can modulate immune effector functions is as intricate as seen by receptors of higher vertebrates (i.e., FcRL5, DNGR-1, and SLAM9). This is evident by the presence of ITIMs and ITSMs within the CYT region of both receptors, the ability to inhibit cell-mediated cytotoxicity when transfected in a cell of lymphoid origin (mouse spleen-derived cytotoxic lymphocytes) while also inducing phagocytosis when transfected in a cell of myeloid origin (RBL-2H3). It is also worth noting that the phagocytic phenotype initiated by IpLITRs was examined in detail using microscopy, and it was shown that IpLITR 1.1b triggered a unique mode of bead capture and engulfment. This involved the formation of elongated filopodia-like projections from the cell surface to connect with targets, a phenotype that was not seen with IpLITR 2.6b, which completely engulfed bead targets in a classical fashion (Lillico et a., 2015). Moreover, IpLITR 1.1b demonstrated a ‘stalled’ phagocytic phenotype where most events (~44% of cells observed) showed beads that were only captured at the cell surface but not fully internalized, in comparison to only ~3% of IpLITR 2.6b-expressing cells showing this stalled phenotype. Additionally, the phagocytosis induced by IpLITR 1.1b was unaffected by incubation at lower temperatures (22°C). This further distinguishes the phagocytic behaviour of IpLITR 1.1b from that of IpLITR 2.6b, which was unable to engulf bead targets below 27°C. Overall, the ability of IpLITR 1.1b to initiate an ITAM-independent mode of phagocytosis represents the first account of a teleost ITIM-containing receptors functional versatility.

In an attempt to explain the underlying mechanisms for IpLITR 1.1b's' unique mode of phagocytosis, our lab focused on the duality of ITSMs, and to a lesser degree, ITIMs, in regulating immune functions. The initiation of signalling responses in cells has been shown to involve SHP-2, which serves as a scaffold for the binding of molecules such as growth factor receptor-bound 2 (Grb2) when recruited to receptor ITIM and/or ITSM (Li et al., 2003; Vemulapalli et al., 2021). From this point, Grb2 can interact with Grb2-associated binders (Gabs), which, when phosphorylated, can associate with PI3Ks, leading to further recruitment and activation of molecules that trigger the phagocytic response (Gu et al., 2003). Alternatively, it has been suggested that Syk can attach to the CYT region of receptors following the phosphorylation of two sequential ITIMs to prompt stimulatory signal transduction, as was observed with platelet endothelial cell adhesion molecule-1 (PECAM-1) (Wang et al., 2011). Conversely, Syk can use one of its two SH2 domains to bind a tyrosine residue (i.e., ITSM or HemITAM) on one receptor and its second SH2 domain to bind another nearby receptor (i.e., aggregated in the lipid raft) holding the same tyrosine residue, leading to downstream signal transduction. This was observed for C-type lectin-like receptor 2 (CLEC-2) (Mori et al., 2012), which is involved in blood coagulation, angiogenesis, and inflammation, and also for DNGR-1, to mediate antigen presentation on professional DCs (Cueto et al., 2020). These hypotheses, based on Syk involvement, were indeed reinforced in additional pharmacological studies where IpLITR 1.1b-mediated phagocytosis was notably reduced by SFK, Syk, and F-actin inhibitors (Lillico et al., 2015).

As pointed out earlier, IpLITR 1.1b suppressed NK-like cell cytotoxicity via phosphorylation and recruitment of signalling molecules to its CYT proximal and distal regions (Montgomery et al., 2012). Given that IpLITR presents such a unique ITAM-independent

phagocytosis method, it was suggested that both the proximal and distal areas of the CYT might also be involved in controlling signalling mechanisms for actin polymerization. Notably, a consensus sequence for the binding of the non-catalytic region of the tyrosine kinase adaptor protein (Nck) was found within IpLITR 1.1bs proximal CYT region (Montgomery et al., 2009), leading to the hypothesis that Nck may function in connecting the Wiskott-Aldrich Syndrome Protein (WASP)- family verprolin-homologous protein-2 (WAVE2) complex, thus triggering the F-actin polymerization machinery (Pils et al., 2012). To investigate whether the proximal and distal region of the IpLITR 1.1b CYT recruits different intracellular molecules, co-immunoprecipitation studies were performed. These studies confirmed that Nck1, Csk, Grb2, and Vav1 were associated with the proximal region of IpLITR 1.1b, while Syk, PI3K, and SHP-2 were linked to the distal region of the membrane CYT (Zwozdesky et al., 2017). Observing the distinctive filopodia-like structures formed by IpLITR 1.1b expressed in RBL-2H3 cells revealed that Nck co-localized with these protrusions, unlike phosphorylated Syk (pSyk) (Lillico et al., 2020). Although the exact functional significance of these extensions remains unclear, the fact that they seem to enhance target engagement suggests a level of basal phosphorylation of IpLITR 1.1b that permits Nck binding, potentially leading to the recruitment of F-actin machinery to generate these structures. Notably, both pSyk and Nck were found at points of bead-cell contact during all phases of phagocytosis, indicating their integral role in the phagocytic process, from target capture to engulfment. Taken together, the association with different signalling molecules suggests that not only can the membrane distal region of IpLITR 1.1b potentially bind to Syk to initiate an ITAM-independent process for cellular activation, but the membrane-proximal region also plays a significant role in enabling these functional responses. Ultimately, IpLITR 1.1b

demonstrates the functional plasticity of this receptor family, and that immunoregulation can be modulated via the same receptor construct to induce different outcomes.

2.5 Goldfish (*Carassius auratus*) LITRs

2.5.1 Discovery and Expression Analyses.

While the catfish system has generated a considerable amount of insight into teleost immunoregulatory receptor-types, our lab was interested in investigating other teleost models that can offer a range of LITR proteins to examine the complexity of immunoregulation in earlier vertebrates. The goldfish transcriptome, previously generated in our lab, was used to identify a truncated sequence for a putative CaLITR, using sequences of IpLITR1, IpLITR2, and IpLITR3 as queries in a Basic Local Alignment Search Tool (BLAST+) (Wang et al., 2020). Subsequently, the goldfish reference RNA database was used to find potential CaLITRs with complete open reading frames using the BLAST algorithm on NCBI. This approach was successful as it resulted in identifying three putative CaLITR sequences, which were renamed CaLITR1, CaLITR2, and CaLITR3. Using the same strategy, CaLITR4, CaLITR5, and CaLITR6 were identified next by searching the goldfish reference RNA database using the CaLITR2 and CaLITR3 cDNA sequences as templates (Wang et al., 2020)

To verify the gene expression of *litrs* in goldfish tissues and immune cells, reverse transcriptase (RT)-PCR experiments were conducted with primers designed to amplify the complete open reading frames of each predicted sequence. Both goldfish *litr2* and *litr3* were found in the goldfish liver, spleen, kidney, heart, brain, gills, intestine, and muscles (Wang et al., 2020). Interestingly, *litr1* expression was not observed in any of the examined tissues. The *litr3*-specific primers yielded two amplified products. One band corresponds to the expected size of CaLITR3 (i.e., 846 bp), while another smaller band (i.e.,597 bp) matched that of a CaLITR3

variant that lacks the D4 domain. To determine if goldfish *litrs* were expressed in goldfish myeloid cells, their transcripts in primary neutrophils from goldfish and during primary kidney macrophage (PKM) development were examined. The transcripts of *litr1*, *litr2*, and *litr3* were all detected in freshly isolated kidney leukocytes and throughout PKM development. Furthermore, all three *litrs* are expressed by primary kidney neutrophils in goldfish (Wang et al., 2020). Taken together, the expression of CaLITRs in several different tissues and in developing leukocytes of myeloid and lymphoid origins that carry immunological importance suggests that these receptors might play a role in goldfish immunity.

2.5.2 Structural Characterization of CaLITRs

A total of 37 CaLITRs, each with distinct extracellular and intracellular domain structures, have been discovered (Wang et al., 2020). The extracellular domains of CaLITRs are distantly related to those found in FcRLs of higher vertebrates, as discussed previously in section 2.4.2. In terms of their intracellular structures, CaLITR1 has a short CYT region and a neutrally charged TM. CaLITR 2.0 contains, what was initially thought to be an ITAM-like sequence ($ExxYxxI_{x25}YxxL$) within its CYT, suggesting that it could be a putative activating receptor. However, functional examination of CaLITR 2.0-mediated phagocytosis (Chapter IV), and subsequent sequence analysis via comparison to consensus tyrosine-based signalling motifs in the literature suggests that the first tyrosine motif (LxYxxI) resembles an ITIM, and the second resembles an ITSM (TxYxxL). CaLITR3 is a putative activating receptor-type featuring a positively charged histidine residue in its TM segment. Both CaLITR4 and CaLITR5 are receptor-types with varying signalling potentials, as they each encompass numerous tyrosine-based motifs within their CYT regions. For instance, the CYT region of CaLITR4 contains a potential endocytic motif, an Nck recruitment motif, an ITAM-like motif that mirrors the one

found in CaLITR2, and an ITIM motif. Similarly, CaLITR5 includes all the motifs seen in CaLITR4 (except for the endocytic motif), but also incorporates a predicted STAT recruitment motif (YxxT). This collection of multiple tyrosine-based motifs in the same CYT region of one receptor resembles that of IpLITR1.1, which was shown to exhibit functional plasticity when transfected in different cell types.

Adding to the diversity and intrigue of this receptor family, five distinct arrangements of distal Ig-like domains in CaLITRs were observed, implying that these receptors may interact with different ligands and employ different strategies and/or varying affinities for external targets (Wang et al., 2020). On top of discovering six CaLITR sequences, our lab also identified variant forms of these receptors. These CaLITR variants share highly similar amino acid sequences with their prototype counterparts, mainly differing in the presence or absence of entire Ig-like domains, TM segments, or CYT regions. For example, the translated sequences of CaLITR3.1 and CaLITR3.2 appear to be exon-skipped splice variants of CaLITR3, containing a variant D1 domain and an added variant D1 domain relative to CaLITR3, respectively. Similarly, CaLITR4.1 lacks the D3 domain of CaLITR4, likely signifying another exon-skipped protein isoform. Transmembrane receptors ability to selectively include or exclude exons encoding extracellular domains is a commonly seen phenomenon in immunity (Martinez & Lynch, 2013; Sahoo & Im, 2010), and is suggested to play a role in fine-tuning immune responses (Lynch, 2004). A well-documented example of an immune receptor that displays extracellular domain splicing to regulate immune functions is CD45, a critical participant in T-cell immunity (Zikherman & Weiss, 2008). In the channel catfish, for example, CD45 also has alternatively spliced forms, similar to the phenomenon seen in other species, although their roles are still unclear (Kountikov et al., 2005; Kountikov et al., 2010). Various other members of the

IgSF family also exhibit alternative splicing in their extracellular regions, including FcRLs (Kulemzin et al., 2011), KIRs (Bruijnesteijn et al., 2018), Down syndrome cell adhesion molecule (Dscam) (Kurtz & Armitage, 2006), receptor for advanced glycation end products (RAGE) (Kalea, 2011), and Carcinoembryonic antigen-related cell adhesion molecule 1 (CAECAM1) (Mißbach et al., 2018). As with other soluble IgSF receptors like LILR (Jones et al., 2009) and cytokine receptors such as IL-1RII (Mantovani et al., 2001), these soluble forms of CaLITRs might exist to provide a negative feedback mechanism to regulate immune responses. Intriguingly, a soluble human FcRL receptor (FcRLA) has been shown to interact with intracellular IgA, IgE, IgG, and IgM, suggesting a potential role in aiding the assembly of these intracellular antibodies (Rostamzadeh et al., 2018). Given that the distal Ig-like domains of CaLITRs are distantly related to mammalian FcRLs, these soluble CaLITR-types may also function as intracellular receptors.

Compared to CaLITR variants with variable extracellular domains, only one putative CaLITR splice variant in its CYT was observed. CaLITR2.1 appears to be a cytoplasmic splice variant of CaLITR 2.0, with a CYT segment deletion of 29 amino acids; taking out a membrane distal tyrosine (Y) motif potentially involved in cell signalling (Wang et al., 2020). Given that CaLITR 2.1 is missing one of the tyrosine motifs found in the CaLITR 2.0 CYT region, this eliminates a tyrosine phosphorylation site, potentially changing how this variant might interact with certain downstream signalling molecules (e.g., Syk, SHP-1, and SHP-2). It is also important to note that the reverse is possible in which CaLITR 2.0 contains an insertion of 29 amino acids. Regardless of the exact mechanism that resulted in the creation of those nearly identical proteins, CaLITR 2.0 and 2.1 present a golden opportunity to examine the functional consequences of CYT-region modification and how this may fine-tune the immunoregulatory potential of LITRs.

Splicing occurrences in the cytoplasmic (CYT) domain of transmembrane receptors are uncommon. For instance, a study of KIR transcripts from 15 individuals revealed just two splicing incidents in the CYT area among 29 unique KIR splicing events (Bruijnesteijn et al., 2018). Allogeneic stimulation of catfish T cells (i.e., TS32.15 and TS32.17 cell lines) induced the production of 16 splice variants of IpLITR 1.1 and IpLITR 2 (Stafford et al., 2006). The majority showed variations in the extracellular region, while only two variants showed variations in the CYT region. In addition, those findings showed that IpLITRs are inducible, and spliced forms are generated in response to alloantigen stimulation of specialized immune cells. An indicator of the potential physiological relevance of splicing events in the native fish context.

Preliminary sequence analysis of CaLITR 2.0 has shown a potential ITAM-like motif ([E]xxY^{465}xx[I]x$_{25}$Y^{494}xx[L]) with an unconventional 25 amino acids gap between the two motifs, while typically, ITAMs are spaced only 6-12 amino acids apart (Billadeau & Leibson, 2002). Consequently, I hypothesize that CaLITR 2.0, when expressed on the surface of AD293 cells and interacting with mAb-opsonized beads, will trigger phagocytosis through ITAM-like dependent signalling pathways. In contrast, the splice variant CaLITR 2.1, which only contains an ITSM, is expected to exhibit reduced phagocytic activity compared to 2.0. This splicing alters the CYT domain, distinguishing almost identical receptors by replacing an ITAM-like motif with an ITSM, leading to possible functional differences. Examining the functional consequences of this CYT-region modification is the objective of this thesis.

CHAPTER III

MATERIALS AND METHODS

3.1 Cells, antibodies, and constructs

3.1.1 Cells

AD293 cells, a variant of HEK-293 cells known for their improved adhesion characteristics, were cultured in Dulbecco's Modified Eagle Media (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA), 100 units/mL of penicillin, 100 mg/mL of streptomycin (Gibco; Thermo Fisher Scientific). For optimal growth conditions, cells were incubated at 37°C and 5% CO_2 in a 75cm^2 vented culture flask. Once at ~80% confluency, culture media was aspirated, and cells were washed with 5 mL of prewarmed Dulbecco's phosphate-buffered saline (DPBS). DPBS was removed, and cells were washed with 2mL of 0.05% trypsin + EDTA and incubated at 37°C, 5% CO_2 for 5 minutes. Cells were then resuspended with 5 mL fresh culture media, and 1 mL of cell suspension was then transferred to a new vented flask containing 9 mL of fresh culture media.

3.1.2 Antibodies

To detect and engage CaLITR constructs on the surface of AD293 cells, the following antibodies were used: Mouse anti-HA mAb IgG_1 (Invitrogen; Thermo Fisher Scientific, Waltham, MA), Mouse IgG_1 isotype (Invitrogen), AF647-conjugated Rabbit-anti-Mouse (H+L) pAb (Invitrogen), PE-conjugated Goat-anti-Mouse (H+L) F(ab')2 (Invitrogen).

3.1.3 Native Constructs

Goldfish cDNAs containing CaLITRs 2.0, 2.1, 4.1, and 6.1 were obtained from another graduate student (Wang et al., 2020). pDisplay, a mammalian expression vector, was used to generate N-terminal hemagglutinin (HA) epitope-tagged constructs, as previously described (Fei et al., 2020). Briefly, oligo primers (Table 3.1; IDT) were designed against CaLITR sequences with the addition of flanking restriction sites (SmaI/PstI) and were used in reverse transcriptase PCR (RT-PCR) reactions. RT-PCR reactions were set up as follows: 0.1 µL of Phusion High-Fidelity DNA polymerase (Invitrogen), 1 µg of provided goldfish cDNA as template, 1 µL of each flanking primer (10 mM), 0.4 µL of 10 mM dNTPs, 4 µL of 5X Phusion HF buffer (Invitrogen), nuclease-free H_2O (IDT) to create a total mixture volume of 20 µL. For all PCR reactions, thermocycler conditions were as follows: 1) 98°C for 1 minute for DNA denaturation, followed by 35 round cycles of 2i) denaturation at 98°C for 15 seconds, 2ii) annealing of DNA at 60°C for 30 seconds and 2iii) extension at 72°C for 1 minute. After cycling, 4) extension took place at 72°C for 10 minutes. PCR products were digested with SmaI and PstI restriction enzymes (FastDigest; Thermo Fisher Scientific) by mixing 1 µg of PCR product, 1 µL of each enzyme, and 2 µL of 10X FastDigest Reaction Buffer for a total of 20 µL. The reaction mixture was then incubated at 37°C for 30 mins. Digested products were run in a DNA electrophoresis gel; target bands were extracted using QIAquick Gel Extraction Kit and ligated into pDisplay mammalian expression vector (Invitrogen; Thermo Fisher Scientific). All plasmid constructs were subsequently confirmed for the presence of CaLITR constructs by Sanger Sequencing at the Molecular Biology Service Unit (MBSU) at the University of Alberta.

3.1.4 Chimeric Constructs

Chimeric constructs (will be referred to as C-CaLITR) were used in this thesis due to the inability of AD293 cells to express native CaLITRs. Because we are only interested in studying the function of CaLITR's signalling region (CYT), the DNA sequence encoding the extracellular Ig-domain and transmembrane (TM) domain of IpLITR 2.6b (Cortes et al., 2012) were merged into the cytoplasmic (CYT) tail of: 1) CaLITR 2.0, 2) CaLITR 2.1, 3) CaLITR 6.1, to create three unique chimeric constructs. These constructs were then sent to GenScript Biotech Corp for synthesis. Received in pUC57 plasmids, these constructs were amplified with restriction sites for SmaI and SalI using oligo primer (IDT; Table 3.1). Amplification using RT-PCR, digestion, and ligation into pDisplay was done as described in section 3.1.3. All plasmid constructs were subsequently confirmed for the presence of Chimeric CaLITR by Sanger Sequencing at the Molecular Biology Service Unit (MBSU) at the University of Alberta.

3.2 Generation of AD293 cell lines stably expressing chimeric CaLITR constructs

3.2.1 Transfection of AD293 cells

$2x10^5$ parental AD293 cells were seeded into a 24-well culture plate in DMEM -/- supplemented with 10% heat-killed FBS. The following day 0.25 µg, 0.5µg, 1µg and 2µg of each CaLITR containing plasmid (C-CaLITR 2.0, C-CaLITR 2.1, C-CaLITR 6.1) were diluted in Opti-MEM reduced serum media (Gibco; Thermo Fisher Scientific), in addition to 1 or 2 µL of Turbofect reagent (Fisher Scientific) for a total volume of 100 µL. pDisplay plasmid containing IpLITR 2.6 was used as a positive control for transfection. Transfection mixtures were mixed by pipetting up and down and were left to incubate at room temperature for 20 minutes. Then they were added dropwise, separately, to each well containing parental AD293 cells. Transfected cells were incubated at 37°C and 5% CO2 for either 24 or 48 hrs before being screened for cell

surface protein expression as described below in 3.2.2. Different plasmid and reagent amounts, as well as incubation periods, were used to assess optimal conditions for cell surface transient expression of C-CaLITRs.

3.2.2 Detection of surface expression of C-CaLITRs-expressing AD293 cells by flow cytometry.

To evaluate the expression of CaLITRs and C-CaLITRs on the surface of AD293 cell lines; cells were stained for the presence of HA-epitope-tagged receptor expression on their surface. Transfected cells were grown to ~ 80% confluency and were harvested with 0.05% trypsin + EDTA. Resuspended in 1 mL of culture media, 300 µL of cells were transferred to Eppendorf tubes containing 700 µL of FACS buffer (1x DPBS, 0.5% bovine serum albumin, 2 mM EDTA, 0.05% NaN3). Samples were then centrifuged for 3 minutes at 500 x g; the supernatant was aspirated, and samples were resuspended in 50 µL of FACS buffer containing primary antibody (0.1 µg of αHA mAb or IgG1 isotype control). To allow cell-antibody interaction, samples were incubated on ice for 30 minutes and then were subsequently washed with 1 mL ice-cold FACS buffer, centrifuged for 3 minutes at 500 x g, decanted, and were resuspended again with 50 µL of FACS buffer containing 0.25 µg of secondary antibody (PE-conjugated goat-α-mouse F(ab')2). Staining and washing steps, as described above, were repeated, and samples were resuspended in 300 µL of FACS buffer. The Attune NxT Flow Cytometer (Thermo Fisher Scientific, Waltham, MA) was then used for analysis using the following gates: 1) stained cells were gated based on FSC-A versus SSC-A parameters to remove large aggregates and cellular debris, 2) FSC-A was gated versus FSC-H to remove doublets and 3) protein expression was gated based on the level of PE fluorescent intensity (indicative of HA-epitope tagged receptor) and compared to IgG1 stained isotype controls.

3.2.3 Sorting and selection of AD293 cell lines positively expressing C-CaLITR constructs.

C-CaLITR-expressing cells were sorted using fluorescence-activated cell sorting (FACS) technology. 48 hrs post-transfection, cells were washed with DPBS and 0.05% trypsin + EDTA. Resuspended in 1 mL of culture media, 500 µL were moved to Eppendorf tubes containing 500 µL of sorting buffer (1x DPBS, 0.5% bovine serum albumin; BSA, 2 mM EDTA). Samples were then centrifuged for 3 minutes at 500 x g; the supernatant was aspirated, and the cell pellet was resuspended in 50 µL sorting buffer containing primary antibody (0.1 µg of αHA mAb or IgG1 isotype control). Samples were then incubated on ice for 30 minutes, subsequently washed with 1 mL ice-cold sorting buffer, centrifuged for 3 minutes at 500 x g, decanted, and were resuspended again with 50 µL of sorting buffer containing 0.25 µg of secondary antibody (PE-conjugated goat-α-mouse F(ab')2). Staining and washing steps, as described above, were repeated, and samples were resuspended in 300 µL of sorting buffer. Cells were then isolated based on their HA-epitope staining expression, and the top 5% of expressing cells were sorted as single-cell clones into a 96-well culture plate using the BD FACS Aria™ III cell sorter. Individual clones were then allowed to grow in DMEM supplemented with 400 ug/mL G418 disulfate salt solution. Once at ~80% confluency, clones were screened for their levels of CaLITR surface expression as described in 3.2.2

3.3 Imaging Flow Cytometry-based Phagocytosis Assays to examine the implication of CaLITRs alternative splicing.

3.3.1 Examining the Phagocytic Potential of C-CaLITR-expressing AD293 Cells

To determine if the C-CaLITRs of interest for this thesis (C-CaLITR 2.0 and its splice variant, C-CaLITR 2.1) can differentially mediate the engulfment of antibody-opsonized beads, the standard protocol used previously in our lab was followed (Fei et al., 2020). Briefly, 4.5 µm

yellow-green (YG) beads (Polysciences, Warrington, PA) were pre-absorbed with protein G (Sigma-Aldrich) and opsonized with 2 μg/mL of α-HA mAb or with 2 μg/mL of IgG_1 isotype control. A day before experiments, $3x10^5$ C-CaLITR (i.e., 2.0, 2.1, 6.1) expressing AD293 cells were seeded in a 24-well cell culturing plate in normal growth media (DMEM, 10% heat-killed FBS, 400 μg/mL G418) and allowed to adhere to the culture plate overnight. C-CaLITR 6.1 contains an activating CYT motif (ITAM) and was previously shown to mediate robust phagocytosis; therefore, it was seeded to be used as a positive control. The following day, culture media was removed and replaced with 500 μL prewarmed (37°C) phagocytosis buffer (1:1 ratio of 1xDPBS with 2 mg/mL bovine serum albumin (BSA) and Opti-MEM). For a 1:3 cell-to-bead ratio, $9x10^5$ opsonized YG beads were added to each sample. Cell plates were then centrifuged at 100 x g for 1 min to synchronize bead-cell interactions, followed by incubation at 37°C for 60 minutes. The phagocytosis buffer was then aspirated, and samples were washed with DPBS, followed by the addition of 200 μL of ice-cold phagocytosis buffer with 0.5 μg of the secondary antibody (AF647 conjugated rabbit-α-mouse pAb) to differentiate surface-bound bead events from phagocytic bead events. Samples were then placed on a shaker at 4°C for 30 minutes. Following that, the antibody mixture was aspirated, and cells were washed with DPBS. Using 500 μL room-temp phagocytosis buffer, the cells were harvested and subsequently transferred to Eppendorf tubes, then centrifuged at 1000 x g for 3 minutes before the supernatant was aspirated. Cells-bead pellets were resuspended with 200 μL of ice-cold 0.05% trypsin + EDTA and incubated on ice for 10 minutes. This is crucial to remove non-specifically bound bead targets from cells. Lastly, 800 μL of ice-cold phagocytosis buffer was added, and each sample mixture was pipetted up and down, followed by centrifugation at 1000 x g for 5 minutes. Samples were then aspirated, and pellets were resuspended in 25 μL of DPBS + 1% paraformaldehyde (PFA)

and were analyzed on the ImageStream mkII (Amnis; Luminex, Austin, TX). 5000 cell events were collected, and data were analyzed using the IDEAS® v6.2 software (Amnis). A connected component masking (Fei et al., 2017) was used to distinguish cells with phagocytosed beads from cells with only surface-bound beads.

Briefly, the component masking methodology works as follows: The secondary antibody stain (AF647) was added to samples to recognize the primary antibodies on the surface of the bead targets. AF647 Rabbit-α-Mouse polyclonal antibodies are large and cannot cross the plasma membrane, therefore, opsonized beads that were fully internalized would not be stained. IDEAS® software allowed for accurate discrimination of bead positioning through a series of masks based on fluorescence, as described in (Fei et al., 2017). Masks for connected components were created to examine the relative size of beads (that is, larger beads were identified as component 1, while smaller beads were identified as component 2, and so on). Only cells containing up to three beads were selected for the analysis since it was found that cells with four or more beads could disrupt accurate fluorescence analysis. Additional masks were employed to measure each detected event by evaluating the relative levels of both green (YG beads) and red (AF647) fluorescence. Based on this analysis, interactions between cells and beads were categorized according to the presence or absence of the 647 fluorescence. Cells that held bead(s) exhibiting red fluorescence were categorized as surface-bound, while cells with at least one bead lacking the red fluorescence were identified as phagocytic.

3.3.2 Examining C-CaLITR-mediated Phagocytosis of Beads with Smaller Size Beads

To determine if the phagocytic phenotype of C-CaLITR 6.1, C-CaLITR 2.0 and C-CaLITR 2.1 expressing AD293 cells are dependent on the target bead size; a phagocytosis flow cytometric assay was conducted similarly to section 3.4.1 except that cell samples were

incubated with 2.0 µm yellow-green bead targets preabsorbed with protein G and opsonized with 2 µg/mL α-HA mAb or 2 µg/mL IgG_1 Isotype control. For the standard ratio of target beads to cells, 1:3 cells-to-beads ratio, $9x10^5$ 2.0 µm opsonized YG beads were added to each sample. To account for the total surface area covered by the beads, like what the 4.5 µm YG beads would hypothetically cover on the surface of AD293 cells, $1.8x10^6$ 2.0 µm opsonized YG beads (1:6 cells-to-beads ratio) were added to each sample of another set of experiments. Samples were analyzed on the ImageStream mkII (Amnis; Luminex, Austin, TX). 5000 cell events were collected, and data were analyzed using the IDEAS® v6.2 software (Amnis). An adjusted connected component masking (Fei et al., 2017) for the 2.0 µm YG beads was used to distinguish cells with phagocytosed beads from cells with only surface-bound beads.

3.3.3 Examining C-CaLITR-mediated Phagocytosis using Higher Concentrations of mAbs on Opsonized Bead Targets.

These sets of experiments were conducted to determine if the phagocytic phenotype of C-CaLITR 6.1, C-CaLITR 2.0 and C-CaLITR 2.1 expressing AD293 cells observed in section 3.4.1 were indeed optimal in terms of the amount of antibodies (2 µg/mL of α-HA mAb or with 2 µg/mL of IgG_1 isotype control) used to opsonize 4.5 µm YG beads. A phagocytosis flow cytometric assay was conducted similarly to section 3.4.1, except that YG bead targets were opsonized with i) 4 µg/mL of α-HA mAb or with 4 µg/mL of IgG1 isotype control or ii) 8 µg/mL of α-HA mAb or with 8 µg/mL of IgG_1 isotype control. As described in 3.4.1, samples were analyzed on the ImageStream mkII (Amnis; Luminex, Austin, TX). 5000 cell events were collected, and data were analyzed using the IDEAS® v6.2 software (Amnis). A connected component masking (Fei et al., 2017) was used to distinguish cells with phagocytosed beads from cells with only surface-bound beads.

3.4 Pharmacological Assessment of CaLITR-mediated Phagocytosis

Imaging flow cytometry was employed to assess pharmacological inhibitors aimed at well-known intracellular signalling molecules. The objective here was to explore the potential involvement and mobilization of specific signalling molecules that are presumably necessary for phagocytosis mediated by C-CaLITRs. $3x10^5$ C-CaLITR (i.e., 2.0, 2.1, 6.1) expressing AD293 cells were seeded in a 24-well cell culturing plate in normal growth media (DMEM, 10% heat-killed FBS, 400 μg/mL G418) and allowed to adhere to the culture plate overnight. The following day, cell media was removed, and samples were washed with DPBS. A phagocytosis buffer containing various pharmacological inhibitors (Table 3.2.) was added, and samples were incubated at 37°C for 1 hour. As a vehicle control, samples were also incubated for 1 hour with 0.5% DMSO. Without removing drug-inoculated phagocytosis buffer, $9x10^5$ of 4.5 μm or 2.0 μm YG beads opsonized with either 2 μg/mL of α-HA mAb or with 2 μg/mL of IgG1 isotype control were added to seeded cell samples and synchronized through centrifugation at 100 x g for 1 minute. As described in 3.4.1, samples were analyzed on the ImageStream mkII (Amnis; Luminex, Austin, TX). 5000 cell events were collected, and data were analyzed using the IDEAS® v6.2 software (Amnis). A connected component masking (Fei et al., 2017) was used to distinguish cells with phagocytosed beads from cells with only surface-bound beads.

3.5 Statistical Analysis

The Shapiro-Wilk test was done to assess the normality of each group. Once the normal distribution of each group was confirmed, the mean of each group -in a similar time point- was compared against all other groups by one-way ANOVA followed by post-hoc Tuckey analysis to determine statistical significance between groups of samples (denoted by alphabetically assigned letters). To determine the significance differences between two groups, a parametric t-test was

used with Welch's correction for unequal variances. All tests were computed using GraphPad, Prism 10 (San Diego, Ca).

Table 3.1 Primers used in PCR experiments.

Primer Name	Sequence 5' – 3'
pDisplay Fwd	TAATACGACTCACTATAGGGA
pDisplay Rvs	ATCCTCTTCTGAGATGAGTTT
CaLITR 2 PstI Fwd	AAACCCATCAAGGTTAATAACTGCAGTGCA
CaLITR 2 SalI Rvs	TGCACTGCAGTTATTAACCTTGATGGGTTT
CaLITR 4.1 SmaI Fwd	TCCCCCGGGACTGTTGAGCCAC
CaLITR 4.1 SalI Rvs	GCGTCGACTTATTATTTAACCTGAGTATAC
CaLITR 6.1 SmaI Fwd	TCCCCCGGGAGCATCAAACCTGCTCAACATGTGT
CaLITR 6.1 SalI Rvs	GCGTCGACTCATCAATGTCCTGATATTTTTTCTTACAGT
C-CaLITR 2 HindIII Fwd	CCCAAGCTTTCCCGGGATGGACGA
C-CaLITR 2 BamHI Rvs	CGCGGATCCCCTTGATGGGTTTCCA

Table 3.2 Pharmacological drug inhibitors, their molecular target and the doses used.

Inhibitor	Target	Dosage (μM)
Cytochalasin D	F-actin polymerization	25
Wortmannin	PI3K PLCγ, MLCK, MAPK	10
PP2	SFK	100
ER 27319	Syk	250

CHAPTER IV

USING PHAGOCYTOSIS ASSAYS TO COMPARE THE IMMUNOREGULATORY POTENTIALS OF GOLDFISH LITR SPLICE VARIANTS

4.1 Introduction

Phagocytosis is generally defined as an actin-dependent cellular engulfment process of particles greater than 0.5 μm induced by various receptor-types (Rosales & Uribe-Querol, 2017). Unicellular organisms use phagocytosis for nutrient acquisition (Uribe-Querol & Rosales, 2017), while in multicellular organisms, phagocytosis is used for defence against invading microbes, as well as the maintenance of tissue homeostasis through the clearance of damaged and dying cells. At the cellular level, phagocytosis is initiated by the engagement of an extracellular ligand to a surface-bound phagocytic receptor. In many instances, this results in the phosphorylation of tyrosine residues within the ITAM of electrostatically recruited adaptor signalling proteins. This leads to the induction of intracellular signalling cascades and activation of the actin polymerization machinery that eventually results in membrane remodelling and internalization of the target (Aderem & Underhill, 1999). Understanding how phagocytosis is regulated and investigating the internal signalling mechanisms that transpire during this process can offer valuable perspectives on how LITRs transduce intracellular signalling events (i.e., their immunoregulatory potentials) with the goal of characterizing how the composition of tyrosine-based motifs within these receptors function to control immune cell responses.

A classic example of receptor-mediated phagocytosis is the well-studied human FcγR and its associated ITAM-containing adaptor FcRγ (Hamerman et al., 2009). Upon binding of IgG-opsonized immune complexes to FcγR, proximal SFKs phosphorylate tyrosine residues within the ITAMs of FcRγ. This creates a high-affinity binding site for the SH2 domain-containing

kinase Syk, which is subsequently activated, resulting in signalling cascade propagation and, eventually, the stimulation of the actin polymerization machinery (Uribe-Querol & Rosales, 2020).

The phagocytic process is a model in our lab to study receptor-mediated immunoregulatory potentials. This effector response is quantifiable with tools such as ImageStream flow cytometry and with accurate resolution using our component masking analysis methods, as described in section 3.4.1 (Fei et al., 2017). The formation of the phagocytic synapse and the recruitment of signalling molecules can then be visualized using standard staining techniques and confocal microscopy. Using the aforementioned techniques, we have explored and documented the role of two functionally unique leukocyte immune-type receptors (IpLITRs) in the channel catfish (*Ictalurus punctatus*) during phagocytosis (Cortes et al., 2014; Lillico at el., 2015). In particular, the ITAM-containing IpLITR 2.6b is an activating type of teleost receptor that facilitates an ITAM-dependent phagocytic response, similar to the conventional stimulatory mammalian Fc receptors (Zhang et al., 2010). This mechanism facilitates the intake of extracellular targets via a Syk-dependent signalling pathway leading to the activation of F-actin polymerization. In comparison, the putative inhibitory receptor IpLITR 1.1b, has been shown to initiate a novel ITAM-independent form of phagocytosis in transfected RBL-2H3 cells (Lillico et al., 2015). However, in contrast to IpLITR 2.6b, this response involves a halted capture and intake phenotype via elongated filopodia-like extensions from the plasma membrane (Lillico et al., 2018). When both IpLITR 2.6b and IpLITR 1.1b were separately transfected into a non-immune mammalian cell line (AD293), IpLITR 2.6b was once again capable of initiating an ITAM-dependent form of phagocytosis, but IpLITR 1.1b failed to internalize targets but it was still capable of capturing the beads (Fei et al., 2016). When both

receptors were co-expressed and subsequently co-engaged, IpLITR 1.1b cross-inhibited the signalling capacity and phagocytic response of IpLITR 2.6b via a SHP-2-dependent mechanism. Notably, the capacity of IpLITR 1.1b to maintain inhibition was achieved through the recruitment of Csk to its membrane-proximal CYT region (Fei et al., 2020).

Recently, the discovery of goldfish (*Carassius Auratus*) (Ca)LITRs present another family of fish immunoglobulin proteins to study immunoregulation. While CaLITRs share phylogenetic, structural, and molecular similarities to receptors belonging to the IgSF, we were particularly interested in a splicing event found in the CYT region of CaLITR transcripts (Wang et al., 2020). Specifically, reverse transcriptase (RT)-PCR and sequence analysis of CaLITR 2.0 and CaLITR 2.1 transcripts, which were isolated from the same fish using the same primers, revealed that these receptors are structurally identical, except for a 29 amino acid deletion in the cytoplasmic tail of CaLITR 2.1. Typically, this occurs at the pre-RNA level, where exons are added or skipped to create different spliced isoforms that may exert different effector functions (Harvey & Cheng, 2016). The ability of transmembrane receptors to selectively include or exclude exons encoding extracellular domains is a commonly seen phenomenon in the immune system and is suggested to play a role in fine-tuning immune responses (Martinez & Lynch, 2013; Lynch, 2004). A well-documented example of an immune receptor that displays extracellular domain splicing to regulate immune functions is CD45, a critical participant in Tcell immunity (Zikherman & Weiss, 2008). However, such splicing events within the CYT region of transmembrane receptors are uncommon. For example, when KIR transcripts were analyzed from 15 human individuals, only two splicing events in the CYT segment were identified out of 29 distinct KIR splicing events (Bruijnesteijn et al., 2018). The main objective of this chapter is to investigate the functional consequences of the alternative splicing event in

the CYT region of CaLITR 2.0, using our established mammalian expression system and the ImageStream flow cytometric phagocytosis assay. Initial sequence analysis of CaLITR 2.0 indicated the presence of an ITAM-like motif ([E]xxY^{465}xx[I]x$_{25}$Y^{494}xx[L]), displaying a nonconventional spacing of 25 amino acids between the two tryosine pockets. Typically, tyrosines within an ITAM are separated by only 6-12 amino acids (Billadeau & Leibson, 2002). Nonetheless, I hypothesized that, when expressed on the cell surface of AD293 cells and engaged with mAb-opsonized beads, CaLITR 2.0 will induce phagocytosis. This predicted response would likely be facilitated via ITAM-like dependent signalling mechanisms. The splice variant, CaLITR 2.1, containing only an ITSM, is predicted to result in diminished phagocytic activity compared to 2.0, a consequence of a CYT-directed splicing event removing a sequence of 29-amino acids including Y^{465}. This CYT modification structurally discriminates the nearly identical receptors from an ITAM-like-containing one to an ITSM-containing variant, and as such, any functional differences can be attributed to this splicing event (Fig. 4.2A). As such, I hypothesize that alternative splicing of tyrosine-containing regions within the CYT region of goldfish LITR2.0 will affect its intracellular signalling capacity as monitored using a phagocytosis assay. Overall, chapter IV will focus on the expression of CaLITR constructs on the surface of AD293 cells and the observed differences in phagocytic activity between CaLITR 2.0 and 2.1 when stimulated under different conditions.

Unexpectedly, CaLITR 2.1 induced significantly higher phagocytic activity at 60 minutes when bound with bead targets. This observation was consistent across different concentrations of antibody used to opsonize bead targets and with beads of varying size. This suggests that the ITSM within CaLITR 2.1 may deploy a unique mechanism to initiate phagocytosis. It also suggests that the putative ITAM-like region within CaLITR 2.0 is not functionally capable of

activating the cells and, more likely, contains an ITIM that suppresses the phagocytic activity observed for CaLITR 2.1. Overall, the results of this chapter provide evidence for how alternative splicing in the CYT region of LITRs may fine-tune their immunoregulatory potentials via the targeted deletion (or insertion) of tyrosine-based motifs.

4.2 Results

4.2.1 CaLITR-Expressing AD293 Cell lines.

CaLITR cDNAs were transfected into AD293 cells as described in section 3.2.1. Non-transfected AD293 cells and cells transfected with an empty pDisplay vector show no expression of an HA-tagged protein on the cell surface (Fig. 4.1A). Native CaLITR constructs (2.0, 2.1, 4.1, and 6.1) also show no expression on the surface of AD293 cells (Fig. 4.1B, 4.1C, 4.1D), whereas the positive control IpLITR 2.6b shows high overall expression levels (Fig. 4.1F). Additionally, cells did not express native CaLITR 2.0 and CaLITR 2.1 under different transfection conditions (either 0.5 or 1.0 μg plasmid DNA, plus 1 or 2 μL of Turbofect) as shown in (Fig. 4.1B and 4.1C). Expression profiles were assessed by staining the cells with either primary anti-HA mAb (green) or isotype control IgG1 (red) followed by PE-conjugated secondary pAb and visualized for their relative fluorescence levels (Fig. 4.1). The x-axis represents the fluorescence intensity associated with the expression level of HA-tagged LITR constructs on the surface of the cell vs. number of cell events (y-axis) measured (Fig. 4.1).

Due to the unsuccessful attempts to express native CaLITRs, chimeric CaLITRs were then created by fusing the extracellular domain and TM domain of the positive transfection control, IpLITR 2.6b, and the CYT region of either: CaLITR 2.0, CaLITR 2.1 or CaLITR 6.1, into a single chimeric construct for each receptor-type. Schematic representations of all receptor constructs used in these experiments are shown in (Fig. 4.2).

Cells transfected with chimeric CaLITR constructs (2.0, 2.1, and 6.1) show high expression of the HA-tagged protein, indicated by a right shift of the PE fluorescence intensity (Fig. 4.1E and 4.1F). C-CaLITR 2.0 construct contains 2 Ig domains, D1 and D2, a TM segment, and a long CYT region containing an ITAM-like motif (Fig. 4.2C). C-CaLITR 2.1, the spliced variant of CaLITR 2.0, contains an identical protein except for a 29 amino acid deletion in its CYT region, removing the ITAM-like motif and leaving an ITSM motif (Fig. 4.2D). For a positive goldfish phagocytic receptor, the ITAM-containing C-CaLITR 6.1 was used in these assays (Fig. 4.2E)

4.2.2 Analysis of CaLITR-mediated Phagocytosis using ImageStream Flow Cytometry

To determine if CaLITR constructs induce phagocytosis, AD293 cells expressing HA-tagged C-CaLITR 2.0, C-CaLITR 2.1 and C-CaLITR 6.1 were incubated with bead targets for 60 minutes and then analyzed using ImageStream flow cytometry. Specifically, 4.5μm YG beads were preabsorbed with protein G, followed by the addition of either 2 μg/mL of αHA or 2 μg/mL of IgG1 isotype. Cells were then incubated with $9.0x10^5$ YG bead particles for 60 minutes. Cells were stained with 0.5 μg secondary antibody (AF647), and finally, cells were fixed and analyzed using an ImageStream Flow Cytometer. Cells were classified as phagocytic if they included at least one fully internalized bead (represented by black bars), or they were considered non-phagocytic if they had one or more beads attached (indicated by grey bars) on their cell surface without any fully engulfed beads. Cells expressing C-CaLITR 6.1 served as a positive control as this construct contains a bonafide ITAM in its CYT region. C-CaLITR 6.1 displayed 93.8% phagocytic activity, a significantly higher value ($p \leq 0.05$) compared to C-CaLITR 2.0 and C-CaLITR 2.1, which showed 16.0% and 41.6% phagocytic activity, respectively, (Fig. 4.3A, 4.3B, 4.3C). These observations were analyzed after 60 minutes of cell-bead interaction. At 60

minutes, the C-CaLITR 2.1 phagocytic activity of 41.6% was significantly higher ($p \leq 0.05$) than the C-CaLITR 2.0 phagocytic activity of 16.0% (Fig. 4.3D). While there was also a significant statistical difference at 30 minutes ($p \leq 0.05$). Both C-CaLITR 2.0 and C-CaLITR 2.1 showed very minimal phagocytic activity at 15 minutes, with 2.9% and 8.1% of sampled cells having internalized beads, respectively. However, the distinction was more apparent at 45 minutes, where 33.5% of cells expressing C-CaLITR 2.1 showed bead engulfment, compared to 13.6% of cells expressing C-CaLITR 2.0 (Fig. 4.3A and 4.3B). Meanwhile, C-CaLITR 6.1 cells displayed 80.8% phagocytic activity at 15 minutes and plateaued with values of 89.1%, 93.7% and 93.8% at 30, 45, and 60 minutes, respectively (Fig 4.3C). As expected, all constructs showed negligible phagocytosis levels when cells were not allowed to incubate with the beads (0 minutes); with values of 1.5%, 2.3% and 2.7% for cells expressing C-CaLITR 2.0, C-CaLITR 2.1 and C-CaLITR 6.1, respectively. All constructs were also incubated with isotype IgG1-opsonized bead targets for 60 minutes to show background phagocytosis levels without direct engagement to the receptors of interest (Fig. 4.3A-4.3C.). Those controls were used in statistical comparisons at each analyzed time point.

4.2.3 CaLITR-mediated Phagocytosis with increasing YG bead αHA mAb Concentrations

To examine if the standard antibody concentration (2 μg/mL αHA) used in the assays above was optimal at resolving the functional difference between C-CaLITR 2.0 and C-CaLITR 2.1, 4.5μm YG beads were also opsonized with either 4μg/mL or 8μg/mL of αHA, and our standard phagocytosis assay was again performed for 60 minutes. Overall, the phagocytic activity of C-CaLITR 2.0, C-CaLITR 2.1, and C-CaLITR 6.1 was similar to that displayed when using the standard concentration of 2 μg/mL αHA (Fig. 4.3A-4.3C). Immediately after the cells were exposed to the beads (at 0 minutes), an expected minimal phagocytic activity was observed

when the C-CaLITR constructs were incubated with the αHA-opsonized bead targets, irrespective of the mAb concentrations (Fig. 4.4A-4.4C). This pattern was evident for all construct-expressing cells, with phagocytosis rates varying from 2.4% to 4.7%, except for an outlier for C-CaLITR 6.1 cells showing 10.4% phagocytic capacity when incubated with 8 μg/mL opsonized beads. This range of phagocytic responses represents the baseline of the assay (Fig. 4.4). At 60 minutes, C-CaLITR 2.0-expressing cells showed a phagocytic activity of 19.4% and 22.3% when incubated with 4 μg/mL and 8 μg/mL αHA opsonized beads, respectively (Fig. 4.4A). A small increase from the 16.0% phagocytic activity that was observed with 2μg/mL of αHA (Fig. 4.3A). Meanwhile, 41.8% and 35.2% of C-CaLITR 2.1-expressing cells showed a phagocytic phenotype at 60 minutes when incubated with 4 μg/mL and 8 μg/mL of αHA-bound YG beads, respectively (Fig. 4.4B). Compared to 41.6% when incubated with 2 μg/mL αHA opsonized beads (Fig. 4.3B). Lastly, cells expressing C-CaLITR 6.1 showed a consistent phagocytic activity of 87.1% and 83.9% when incubated with 4 μg/mL and 8 μg/mL of αHA-bound YG beads, respectively (Fig. 4.4C), compared to 93.8% when incubated with beads conjugated with 2μg/mL of αHA (Fig. 4.3C).

4.2.4 Analysis C-CaLITR-mediated Phagocytosis using 2 μm YG bead targets.

Receptor-mediated phagocytosis of targets with varying sizes requires the recruitment of different signaling molecules and, as such, different overall intracellular mechanisms of target engulfment (Pacheco et al., 2013). To further resolve the functional differences in CaLITR-mediated phagocytosis, smaller YG bead targets (2 μm) were opsonized with the standard concentration of 2 μg/mL αHA mAb, and phagocytosis assays were performed for 60 minutes. Two sets of experiments were conducted: 1) using the standard 1:3 cells to beads ratio, and 2)

1:6 cells to beads ratio. Overall, higher phagocytic activity was observed for each of C-CaLITR 2.0 and CaLITR 2.1 across all time points (except for 0 minutes), when compared to phagocytosis of 4.5 μm bead targets (Fig. 4.3A, and 4.3B). For the 1:3 cells to beads ratio, my results show that 20.6%, 30.7%, 36.2%, and 41.7% of C-CaLITR 2.0 cells exhibit phagocytic activity at 15, 30, 45, and 60 minutes, respectively (Fig. 4.5A). Previously, 2.9%, 8.5%, 13.6%, and 16% (at 15, 30, 45, and 60 minutes, respectively) of C-CaLITR 2.0 expressing cells were able to phagocytose 4.5 μm (Fig. 4.3A). An increase ranging from ~2 to 2.5-fold. In comparison, C-CaLITR 2.1-expressing cells showed a phagocytic activity of 30.2%, 58.0%, 65.4%, and 72.9% at 15, 30, 45, and 60 minutes, respectively (Fig. 4.5C), compared to 8.10%, 19.2%, 33.5% and 41.5% when incubated with 4.5 μm opsonized beads (Fig. 4.3B). An increase ranging from ~1.5 to 3-fold. Surprisingly, cells expressing C-CaLITR 6.1 showed ~0.5x decrease in phagocytosis of smaller beads, as they engulfed 62.8%, 73.4%, 78.6% and 82.7% at 15, 30, 45, and 60 minutes, respectively (Fig. 4.5E). To verify that this phenotype is CaLITR-mediated and not a function of other receptors expressed by AD293 cells (e.g., scavenger receptors), the same constructs were incubated with beads opsonized with 2 μg/mL IgG1 isotype (Fig. 4.5), and indeed, minimal phagocytosis (ranging from 3.4% to 14.5%) was observed for all constructs.

For the experiments examining the 1:6 cells to beads ratio, similar trends to the assays of the 1:3 cells to bead ratio were observed (C-CaLITR 2.0: 3.7%, 20.2%, 29.5%, 38.4%, 41.4%; C-CaLITR 2.1: 5.0%, 33.4%, 57.0%, 64.2%, and 70.9%, at 0, 15, 30, 45, and 60 minutes, respectively; Fig. 4.5B, and 4.5D). However, C-CaLITR 6.1 showed another decrease in phagocytic capacity as only 3.5%, 54.6%, 64.9%, 68.3%, and 76.2% of cells were seen with at least one engulfed bead target (Fig 4.5F).

4.3 Discussion

4.3.1 Overview

In this chapter, my primary goal was to explore the functional differences induced by two nearly identical receptors, CaLITR 2.0 and CaLITR 2.1, with the structural difference being a CYT region splicing event that removes a 29-amino acid sequence, including a tyrosine residue (Y^{465}), in the latter. I set up a heterologous expression system, which requires transfecting and expressing CaLITR constructs (cloned in a pDisplay vector that attaches an N-terminal HA tag to the translated protein) on the cell surface of AD293 cells. I then engaged and activated the constructs using beads that were opsonized with anti-HA mAb and measured their phagocytic activity with ImageStream Flow Cytometry. To my surprise, native goldfish LITR constructs were not expressed on the surface of AD293 cells, a phenomenon that was not seen previously using catfish and zebrafish LITRs (Wang et al., 2023). I then created chimeric CaLITR constructs, which allowed me to express my proteins of interest (i.e., C-CaLITR 2.0 and C-CaLITR 2.1). While they do not completely resemble the native CaLITR constructs, they still contain the exact same CYT regions as their native counterparts and contain the site of alternative splicing that my project was focused on. Overall, my findings suggest that: 1) Select native goldfish LITRs cannot be expressed on the cell surface of AD293, 2) C-CaLITR 2.1, triggers a delayed form of phagocytosis that is more pronounced than that observed with C-CaLITR 2.0. This suggests that alternative splicing of the mRNA could be a mechanism used to fine-tune immune effector functions. Additionally, my results suggest that an ITSM motif (present in C-CaLITR 2.1) can modulate a phagocytic response, albeit less potently than the more extensively researched ITAM-dependent phagocytic response observed by C-CaLITR 6.1.

4.3.2 The inability to express native CaLITR constructs.

The prior use of mammalian heterologous expression systems in our laboratory has facilitated the investigation of the phagocytic properties of IpLITRs and further analysis of the associated immune response signalling networks (Cortes et al., 2012; Lillico et al., 2015; Fei et al., 2020). Also, the use of imaging flow cytometry has resulted in improved detail regarding the placement of opsonized YG bead targets relative to receptor-bearing cells during phagocytosis. This technique has provided additional insights into the signalling abilities of teleost immunoregulatory receptors. My dissertation work capitalizes on this system, which has proven invaluable in understanding IpLITR immune regulation, including the inhibitory crosstalk between IpLITR 1.1b and IpLITR 2.6b (Fei et al., 2020). This system serves as an effective platform for examining recently discovered CaLITRs, identifying their phagocytic phenotypes, and further investigating the functional significance of alternative splicing, specifically ones that pertain to the receptor region involved in transducing extracellular signals to modulate cellular functions.

A major obstacle faced when establishing this system for goldfish proteins was the inability to express native CaLITR constructs in AD293 cells. CaLITRs 2.0, 2.1, 4.1, and 6.1 were cloned into a mammalian expression vector, pDisplay (Invitrogen; Thermo Fisher Scientific), as described in section 3.1.3. pDisplay includes an upstream Ig Kappa leader sequence, which is a signal peptide that tags the DNA sequence of the target protein to be translocated to the cell membrane. The plasmid also carries a downstream Platelet-derived growth factor receptor transmembrane domain (PDGFR-TM) sequence that helps anchor the target protein, once translated, to the plasma membrane for display (Guerrero-Esteo et al., 2002).

However, all LITR constructs include a stop codon upstream of the PDGFR-TM sequence; thus, the ability of those constructs to anchor themselves to the cell membrane is dependent on their correct placement ahead of the Ig Kappa leader sequence and the plasmid being successfully internalized and integrated by the transfected cell population. AD293 cells transfected with CaLITRs survived the Geneticin (G418, 400 ug/mL) antibiotic selection process, which suggests that the transfected cells were able to integrate, transcribe and translate the plasmid vector. pDisplay contains a Kanamycin resistance gene which confers resistance to Geneticin antibiotics in mammalian cells (Katoh et al., 1987). Before transfection experiments were initiated, the cloned plasmid sequence for each construct was verified (Appendix; Fig. 1), and, indeed, select CaLITR constructs are in the correct frame upstream of the Ig Kappa signal peptide. This confirms that CaLITRs' lack of cell surface expression is not due to cloning or transfection errors and suggests that it could be an inherent property of the selected CaLITR receptors.

Since the cloning and transfection processes were not the issue, I looked at the possibility of cellular trafficking in the context of signal peptides. The transportation of proteins to specific regions of the cell membrane is meticulously controlled at various steps of the cells trafficking system. This regulation process identifies signal peptides within the protein, as it is being processed in the endoplasmic reticulum (ER) and Golgi apparatus, which ultimately decides the destination of the protein (Zarei et al., 2004). Signal peptides are numerous and can vary depending on the cell context. Some include retention signals, which prevent the protein from moving forward. Retrieval signals, which send back proteins to their primary location, and export signals, which facilitate the protein's exit from the ER organelles. Retention and retrieval sequences have been found in several proteins inside the lumen and type I transmembrane proteins (such as proteins of the IgSF) (Ellgaard & Helenius, 2003; Cela et al., 2022). These

motifs primarily consist of KDEL, RRxx, and KKxx protein sequences. As an example, the KDEL motif found in some proteins can be recognized by a KDEL receptor found in the ER. This interaction leads to MAPK pathway activation, ultimately leading to the target protein getting retrieved from the Golgi complex by coatomer protein (COP)I-coated vesicles (Ellgaard & Helenius, 2003; Cela et al., 2022). A similar mechanism was seen with type 1 transmembrane proteins with double arginine (RRxx), or double lysine (KKxx) motifs (where X denotes any amino acid) (Jackson et al., 1990; Nilsson et al., 1989). All CaLITR constructs used in this thesis (2.0, 2.1, 4.1 and 6.1) contain a double arginine in their amino acid sequence, while 2.1 and 4.1 also contain a double lysine. While this may suggest a potential retention sequence causing the lack of expression of CaLITRs in the AD293 system, the transfection protocol positive control, IpLITR 2.6b, contains a double arginine, as well as the zebrafish receptor, DrLITR 1.2, which were both successfully expressed by AD293 cells (Wang et al., 2023). None of the CaLITR constructs contain KDEL sequences. Overall, the reason why native CaLITRs are not being expressed is not clear and requires further investigation. Such potential studies include protein expression in cell lysates of transfected populations, or transfection of CaLITR constructs with mutated retention motifs. Worth noting that since chimeric constructs can be expressed, then it may be the extracellular Ig domains and/or the TM segments of CaLITRs that are responsible for failure to express these receptors. Altogether, since the goal of my research is to resolve the functional differences of the CYT region splicing events, the chimeric constructs created (C-CaLITR 2.0, C-CaLITR 2.1, and C-CaLITR 6.1) were sufficient to circumvent the unexpected surface expression difficulties and provide an opportunity to investigate the CaLITR CYT region immunoregulatory potentials in transfected AD293 cells. Therefore, the discussion below will

focus on the results obtained using surface-expressed chimeric receptors containing CaLITR CYT regions.

4.3.3 Effects of CYT-region Splicing on CaLITR Function

We originally identified five different CaLITRs (1 through 5), with multiple different isoforms for each receptor-type. Structurally, isoforms varied in the type and number of their extracellular Ig-like domains (e.g., CaLITR 4.0 and CaLITR 4.1) or the absence/presence of TM segments and CYT regions creating possible soluble or intracellular forms (e.g., CaLITR 2.3 and CaLITR 2.4) (Wang et al., 2020). The frequent occurrence of transmembrane receptors in the immune system selectively incorporating or excluding exons that code for extracellular domains or entire TM/CYT regions is a well-documented phenomenon, and we speculate that such isoforms differ in their overall functional abilities, which may include ligand specificity or affinity. However, the splicing of CYT regions that contain tyrosine motifs implicated in intracellular signalling is a less common phenomenon, and in the case of CaLITRs, CaLITR 2.1 presents the only identified LITR receptor with splicing in its CYT region (Wang et al., 2023). CaLITR 2.0 was initially thought to contain an ITAM-like motif ([E]xxY^{465}xx[I]x$_{25}$Y^{494}xx[L]), with an atypical 28 amino acid distance between its two tyrosine residues, thus predicting it to be a putatively activating motif, and a phagocytic assay was used to examine its function in comparison to its splice variant, CaLITR 2.1. As such, CaLITR 2.0 was hypothesized to induce a potent phagocytic response when engaged in our heterologous expression system. In contrast, only 16.0% of C-CaLITR 2.0-expressing cells engulfed opsonized bead targets, in comparison to 41.6% of C-CaLITR 2.1-expressing cells (Fig 4.3 A & 4.3B). These results suggested that the two tyrosines, Y^{465} and Y^{494} do not behave like the typical activating ITAM sequence, but most likely function as two distinct motifs, namely an ITIM and an ITSM. Alternatively, the ITSM-

containing splice variant CaLITR 2.1 was predicted to induce little to no phagocytic activity due to the removal of what was thought to be an ITAM-like sequence. Surprisingly, this construct mediated significant phagocytic activity starting at 30 minutes (Fig. 4.3B). Collectively, these results suggest that alternative splicing in the CYT region of CaLITR 2.0, which resulted in the removal of an ITIM, allows phagocytosis via an ITSM-mediated mechanism. It is important to note that the C-CaLITR 2.1/ITSM-mediated phagocytic phenotype is delayed, as very few cells showed engulfment at earlier time points (15 minutes) when compared to an ITAM-containing receptor, like IpLITR 2.6b (Fei et al., 2020). Previously in our lab, C-CaLITR 6.1 was also shown to induce a potent phagocytic response like that of IpLITR 2.6b, hence, this was used as a positive phagocytic receptor in my functional assays. Indeed, cells expressing C-CaLITR 6.1 showed a robust, and quick (as early as 15 minutes) phagocytic activity (Fig 4.3C). Taken together, by comparing the phagocytic capacities observed between C-CaLITR 2.0 and C-CaLITR 2.1, I was able to resolve the functional differences between alternatively spliced CaLITRs. Overall, my results show the first functional characterization of alternatively spliced teleost immune receptors, in which 29 amino acids in the CYT of one receptor were spliced, and consequently leading to a more activating phagocytic phenotype. In addition, my results are the first to suggest that ITSMs can induce phagocytosis in AD293 cells, however, it is less potent than that induced via ITAMs (e.g., C-CaLITR 6.1, IpLITR 2.6b). Interestingly, ITSM-mediated phagocytosis is delayed and occurs slowly, which opens the doors to investigating the signalling mechanisms involved in this novel phenotype.

Experiments were also performed under various conditions to help further resolve the functional differences between C-CaLITR 2.0 and its splice variant, C-CaLITR 2.1. These conditions included: i) increasing the concentration of antibodies used for bead opsonization, and

ii) assessing the effect of bead size on CaLITR-mediated phagocytosis. The first set of experiments was done to examine if the standard concentration of αHA antibodies (2 μg/ml) used in previous and similar assays in our lab was optimal to resolve the phenotypic differences between 2.0 and 2.1. In other words, I examined if the binding avidity of CaLITRs to target beads, specifically through different antibody concentrations, can lead to different phagocytic activity by C-CaLITRs. Avidity is characterized by the cumulative strength of interactions between the receptor and its corresponding ligands, considering both the affinity of the immediate bond made and the total number of interactions between the receptors and ligands (Zhang et al., 2006). Generally, heightened receptor-ligand (or bead) avidity results in increased receptor-related signalling, which facilitates a more robust and swift cellular effector response. For example, murine macrophages were shown to engulf more target particles when they were opsonized with higher concentrations of sheep anti-BSA IgG (Pacheco et al., 2013). Another example is the FcαRI receptor, which associates with the ITAM-containing adaptor molecule FcRγ upon engagement with IgA (Ben Mkaddem et al., 2013). Large immune complexes made up of multiple IgA molecules and ligands produce stronger receptor avidity. Here, the ITAM can be phosphorylated by SFKs, instigating Syk recruitment and subsequent summoning and activation of additional signalling molecules to trigger cellular effector responses like phagocytosis, ADCC, and cytokine release. However, when engaged with monomeric IgA, FcαRI assumes a different function where low valency binding of the ligand leads to Syk-mediated recruitment of SHP-1, which tempers phosphorylation signalling and overall inhibits cell activation (Ben Mkaddem et al., 2013). Therefore, increasing opsonizing-antibody concentrations were analyzed for CaLITR constructs to bead targets to optimally resolve the phagocytic potential for these receptors. Due to the slightly unstable expression profiles of cells

displaying C-CaLITR 2.0, 2.1 and 6.1 constructs, I chose to optimize the assay by increasing the amount of antibodies rather than decreasing. My results showed that both C-CaLITR 2.0 and C-CaLITR 2.1, even with increasing antibody concentrations (4μg/mL & 8μg/mL), induce similar phagocytic activity when compared to the standard concentration (2μg/mL) used in the initial characterization experiments (Fig 4.4). This verifies that the opsonization concentration used initially was optimal to resolve the functional differences between C-CaLITR 2.0 and its splice variant, C-CaLITR 2.1. As a result, I was able to confidently use 2 μg/mL of mAb to opsonize bead targets for all experiments in this thesis.

The second set of experiments utilized bead targets of smaller size (2 μm) to examine if CaLITR-mediated phagocytosis is size-dependent. Phagocytosis is typically driven by the actin cytoskeleton machinery, which supplies considerable force to overcome the physical barriers needed to envelope larger particles (>5μm). This differs from how smaller particles are internalized via endocytosis because phagocytosis doesn't depend on coat proteins such as clathrin and caveolin, and the actin cytoskeleton plays a less significant role in endocytosis (Bohdanowicz & Grinstein, 2013). The polymerization of actin during phagocytosis requires the activity of Rho-GTPases and PI3K-family kinases, which are particularly essential for the phagocytosis of larger particles, those exceeding 5μm. Mechanisms of phagocytosis vary due to the diversity in pathogen sizes and the distinct receptors they interact with. For instance, the larger hyphal forms of the fungus *Aspergillus fumigatus* are consumed through FcR-mediated phagocytosis, while the smaller spores of the same fungus are engulfed via mannose receptors (Heung, 2020). The efficiency of this process depends on other factors such as rigidity and the density of antibodies on the particle surface (Freeman & Grinstein, 2020; Pacheco et al., 2013; Vorselen et al., 2020), as well as the space between the phagocyte’s membrane and the particle

surface (which is determined by the size of the Fc-receptor-ligand complex) (Bakalar et al., 2018). Less rigid apoptotic cells are engulfed with lower efficiency than stiffer targets like bacteria and yeasts (Freeman & Grinstein, 2020). Conversely, the process of phagocytosis by complement receptor 3 (CR3) occurs via loosely formed membrane ruffles controlled by linear actin, smaller membrane protrusions, and phagosomal "sinking" into the cytoplasm (Swanson, 2008). Recent studies reveal that Fc- and complement-mediated uptake can be interconnected, and both may depend on the anchoring of actin through integrin αMβ2 at the leading edge of pseudopods at the phagocytic cup (Freeman & Grinstein, 2020; Jaumouillé et al., 2019). Considering these factors, we control for the amount and geometry of the target particle, as we are using carboxylated spherical beads from the same manufacturers, as well as the density of antibodies on the particle surface (i.e., beads were opsonized with 2 μg/mL mAb). This allows us to directly compare the impact of target size on the phagocytic activity of C-CaLITRs. My results show that there is a significant increase in the percentage of cells (expressing either C-CaLITR 2.0 or C-CaLITR 2.1) internalizing smaller bead targets, 2 μm, compared to larger bead targets, 4.5μm (Fig 4.6A and 4.6B). Specifically, the ITSM-containing splice variant, C-CaLITR 2.1, shows a higher phagocytic activity, but interestingly, a more immediate one (as early as 15-30 minutes) than that observed for its delayed phenotype (45-60 minutes) when larger bead targets (Fig. 4.6A and 4.6B). Background controls showed minimal phagocytic activity for all constructs, verifying that this observation is C-CaLITR-mediated (Fig. 4.5). The existing evidence seems to point to an optimal diameter of ~3μm for spherical particles for the most effective phagocytosis. Studies conducted both *in vivo* and *in vitro* have shown that rat alveolar macrophages can efficiently remove particles within the 1-5μm range (Makino et al., 2003), with the ideal particle size being around 2-3 μm (Hirota et al., 2007). This trend was consistent with

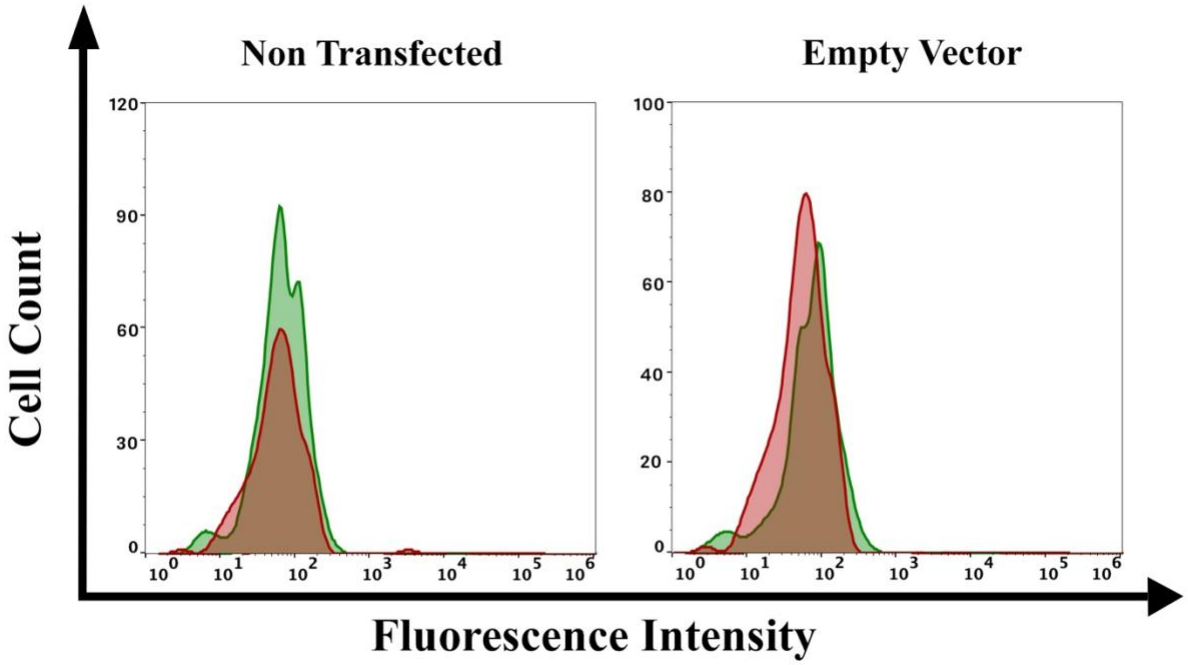
murine macrophages (RAW 264.7) that showed higher phagocytic activity against 0.5μm, 1μm, and 2μm targets compared to relatively low activity when stimulated with 3 μm and 4.5 μm beads (Pacheco et al., 2013). Overall, my results suggest that C-CaLITR 2.1-mediated phagocytosis is size-dependent, in which more cells are engulfing small (2.0 μm) beads than they do large (4.5 μm) beads. The functional distinction between C-CaLITR 2.0 and its splice variant, C-CaLITR 2.1, is consistent with that observed in assays of larger beads, as 2.1 shows a higher phagocytic capacity of small beads. This marks another unique and novel observation of CaLITR-mediated effector function and opens more questions regarding the underlying signalling networks, likely triggered by the lone ITSM motif.

While these findings on the regulation mediated by CaLITRs provide novel insights into how alternative splicing of signalling motifs might fine-tune phagocytosis, it's important to remember that my results depend on the overexpression of CaLITRs in a well-understood mammalian cell line. Although this epithelioid cell line is not an immune cell, it has proven effective in previous studies for characterizing immune receptor-types. Historically, the heterologous expression system provided valuable information on the immunoregulatory potential of Fc receptors (Weinshank et al., 1988; Ravetch & Kinet, 1991). In our lab, the utility of the AD293 system became evident when observations of phagocytosis mediated by IpLITR 2.6b appeared to have similar characteristics to our earlier studies on IpLITR 2.6b within the myeloid immune cell line RBL-2H3. Other studies on crosstalk that used a different heterologous expression system expressed chimeric FcγRIIA receptors (with the extracellular domain of FcγRI and CYT region of FcγRIIA) alongside FcγRIIB. This setup was specifically designed to explore the crosstalk interactions between the two receptors while eliminating unintended interactions with other receptors found on hematopoietic cells (Chan et al., 2011). The reactions

observed through overexpression of these receptors in this model allowed for a conserved ITAM-dependent phagocytosis response within a non-phagocytic cell line. Consequently, investigations into the signalling dynamics governing these reactions can be directly linked to receptors that trigger the activation of these conserved signalling pathways.

In conclusion, I utilized a mammalian heterologous expression system previously established in our lab to investigate the functional implication of alternative splicing found in the CYT region of teleost immune receptors. First, I showed that C-CaLITR 6.1 is analogous to other ITAM-containing immunoregulatory receptors in terms of its bonafide phagocytic potential (Fei et al., 2016; Hamerman et al., 2009), and as such, was used as a positive control for the phagocytosis assays. I also showed that what we thought to be an ITAM-like motif is more likely an ITIM and an ITSM in the CYT region of CaLITR 2.0. In addition, my results showed that the splicing of the ITIM sequence resulted in the amplification of the phagocytic response induced by C-CaLITR 2.1 using both small and large bead targets. Additionally, my results indicated that the ITSM within the receptor tail modulates the signalling ability of the receptor, facilitating a unique form of delayed phagocytosis. However, the same ITSM-containing receptor can modulate a more rapid and robust phagocytic response against small particles, adding another dimension of size-dependency of this phenotype. Overall, these findings suggest that alternative splicing, may serve as a mechanism through which teleost immunoregulatory receptors can fine-tune their immunoregulatory functions. Chapter V of this thesis will delve into the potential signalling components (Syk, PI3K, SFKs, and F-actin polymerization) involved in the unexpected phagocytic response of C-CaLITR 2.1. This will be explored via the pharmacological inhibition of signalling molecules during phagocytosis induction.

A.


Non Transfected
Empty Vector
Cell Count
Fluorescence Intensity
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90
60
30
0
100
80
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40
20
0
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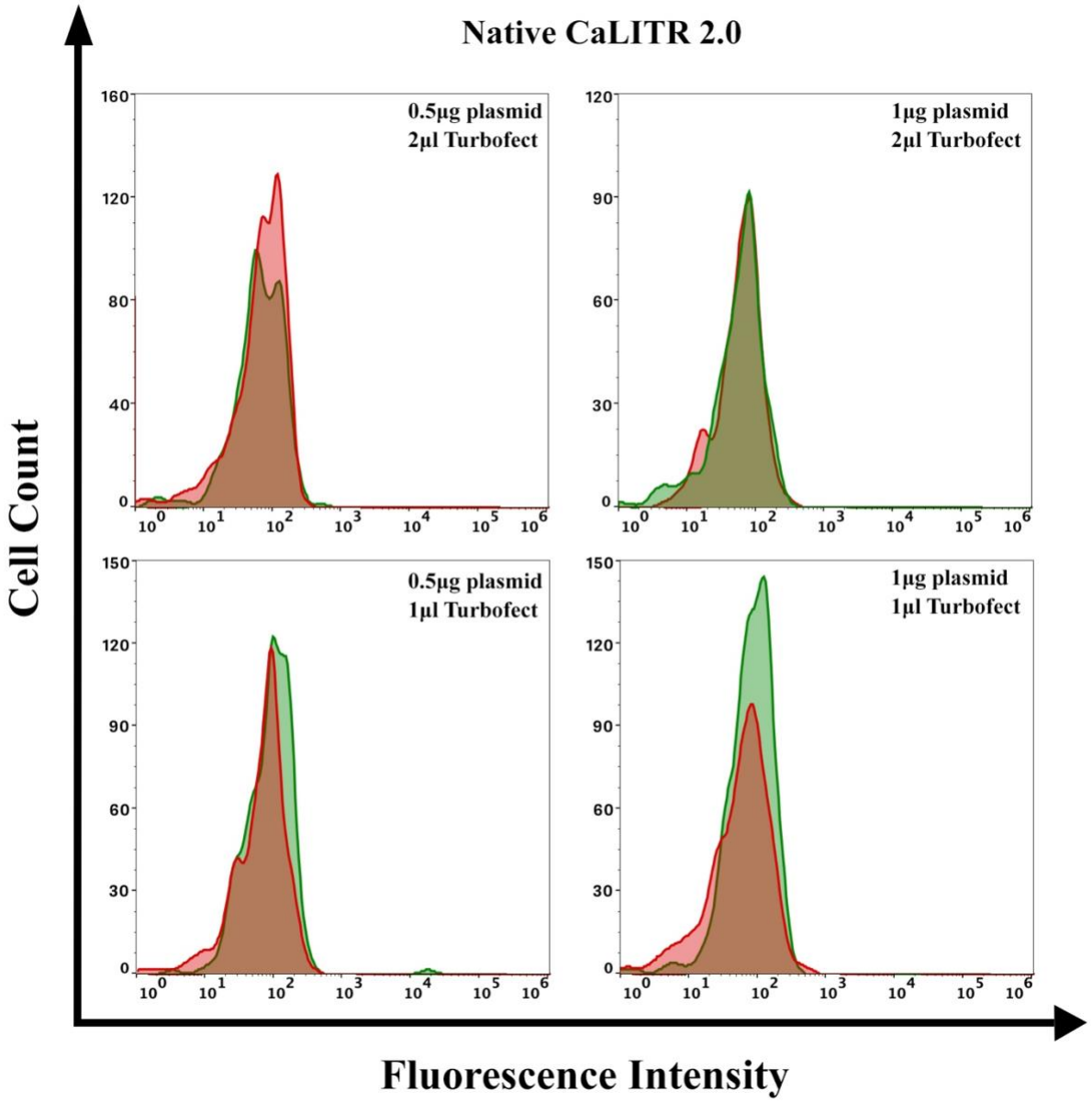
B.


Native CaLITR 2.0
0.5µg plasmid
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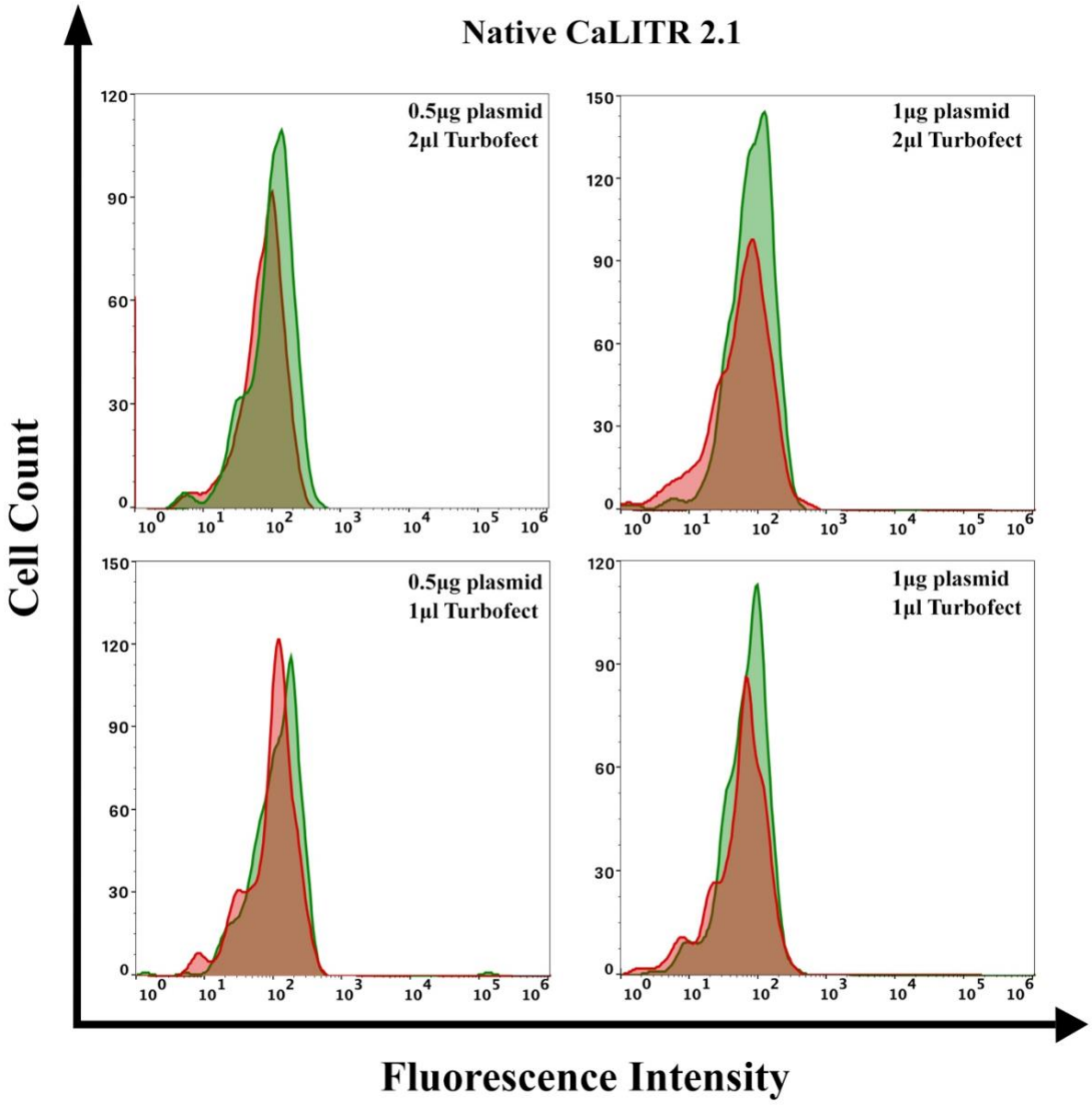
1µg plasmid
2µl Turbofect
0.5µg plasmid
1µl Turbofect
1µg plasmid
1µl Turbofect
Cell Count
Fluorescence Intensity

C.


Native CaLITR 2.1
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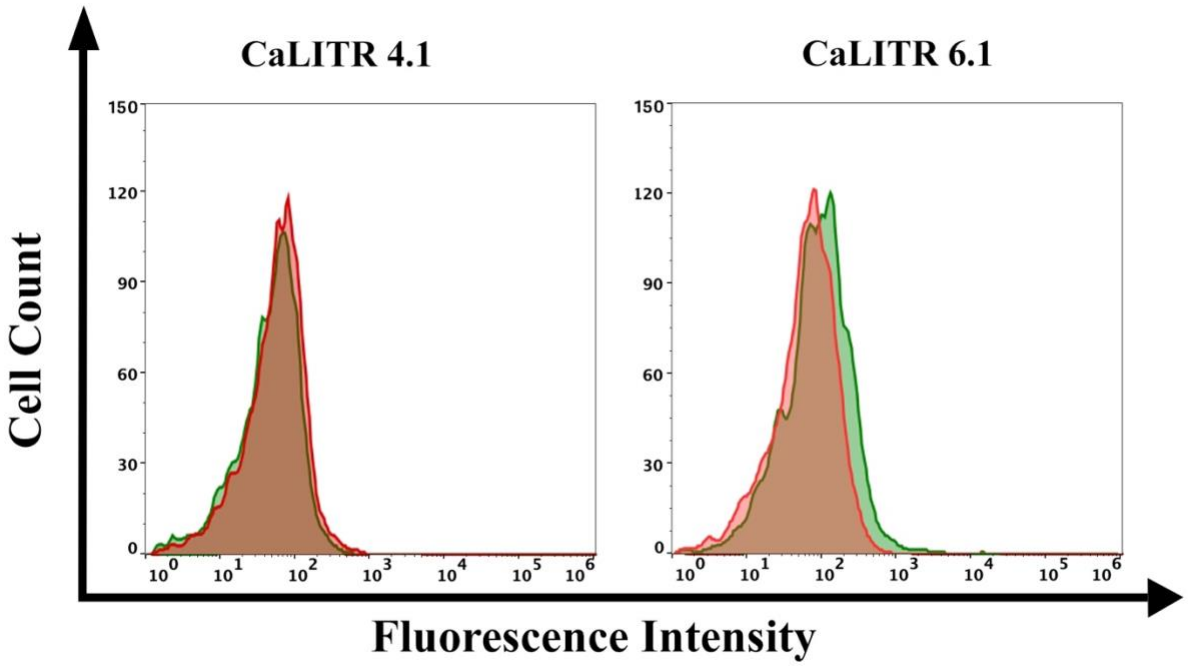
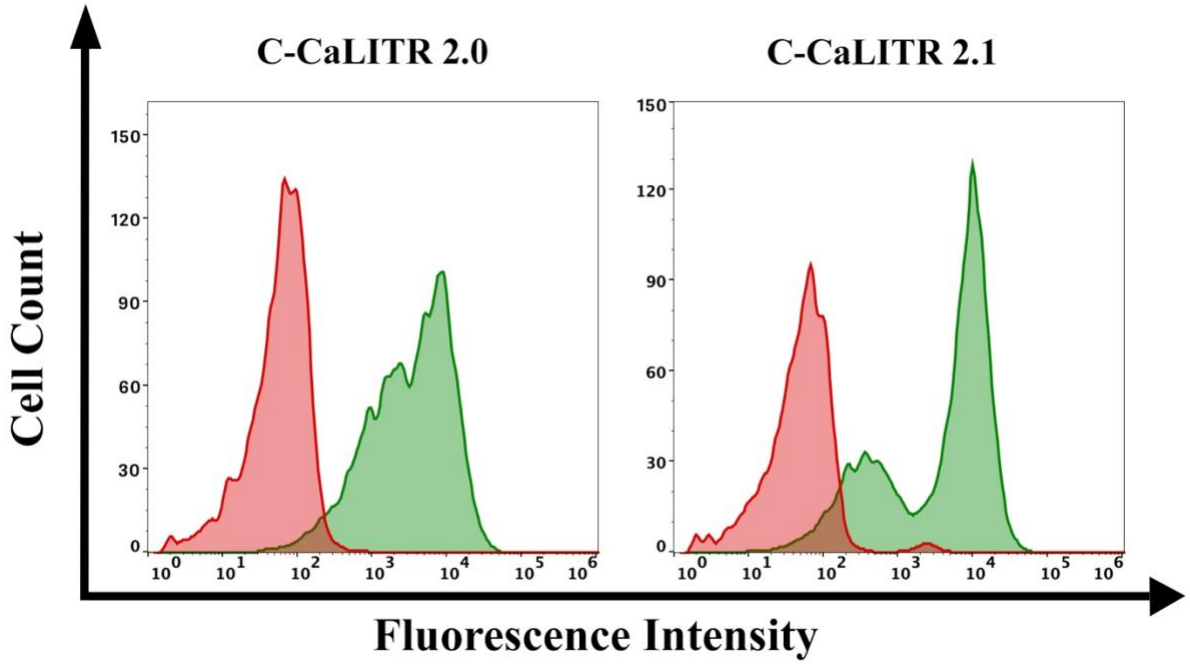
1µg plasmid
2µl Turbofect
0.5µg plasmid
1µl Turbofect
1µg plasmid
1µl Turbofect
Cell Count
Fluorescence Intensity

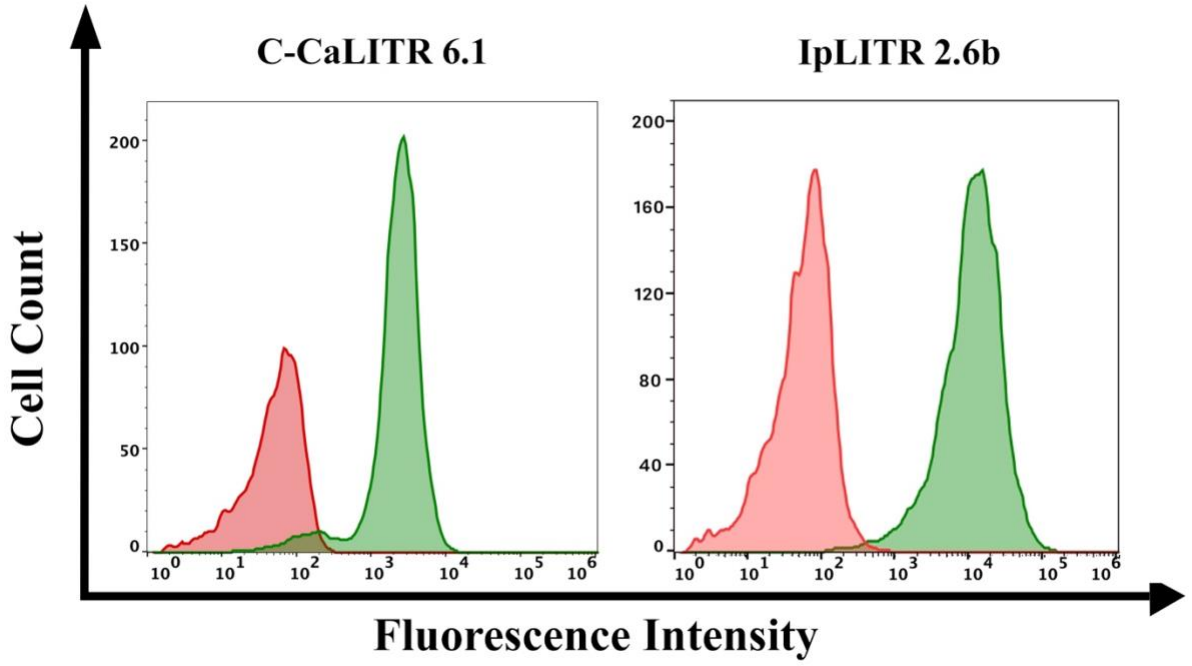
D.


CaLITR 4.1
CaLITR 6.1
Cell Count
Fluorescence Intensity

E.


C-CaLITR 2.0
C-CaLITR 2.1
Cell Count
Fluorescence Intensity

F.


C-CaLITR 6.1
IpLITR 2.6b
Cell Count
Fluorescence Intensity
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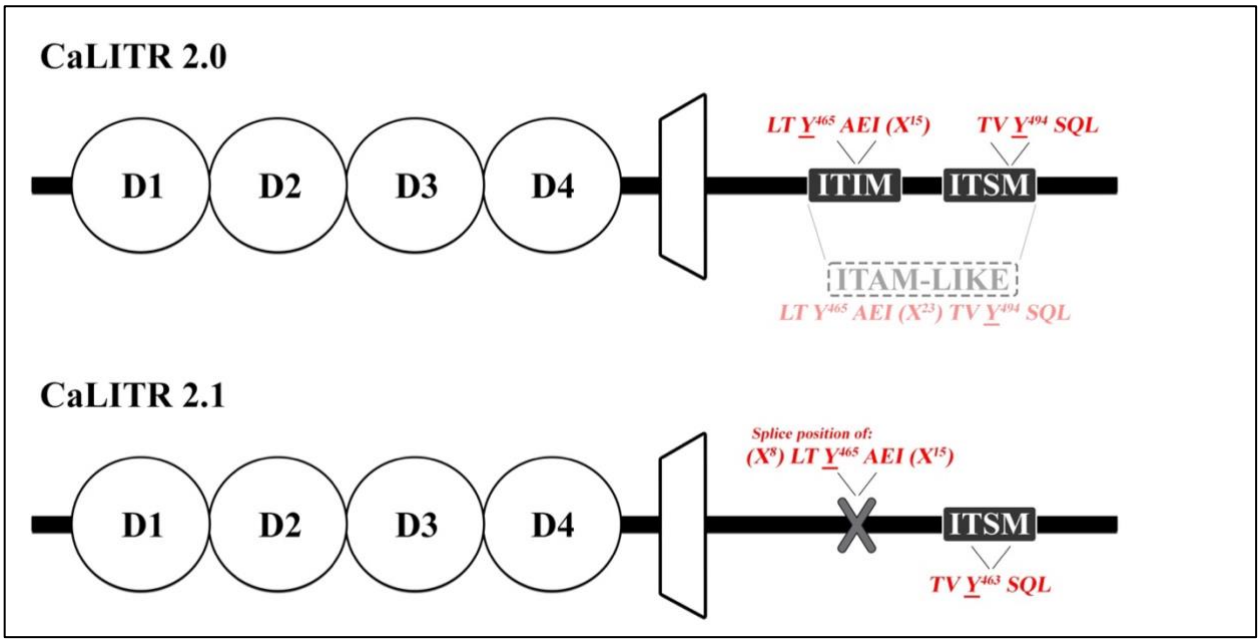
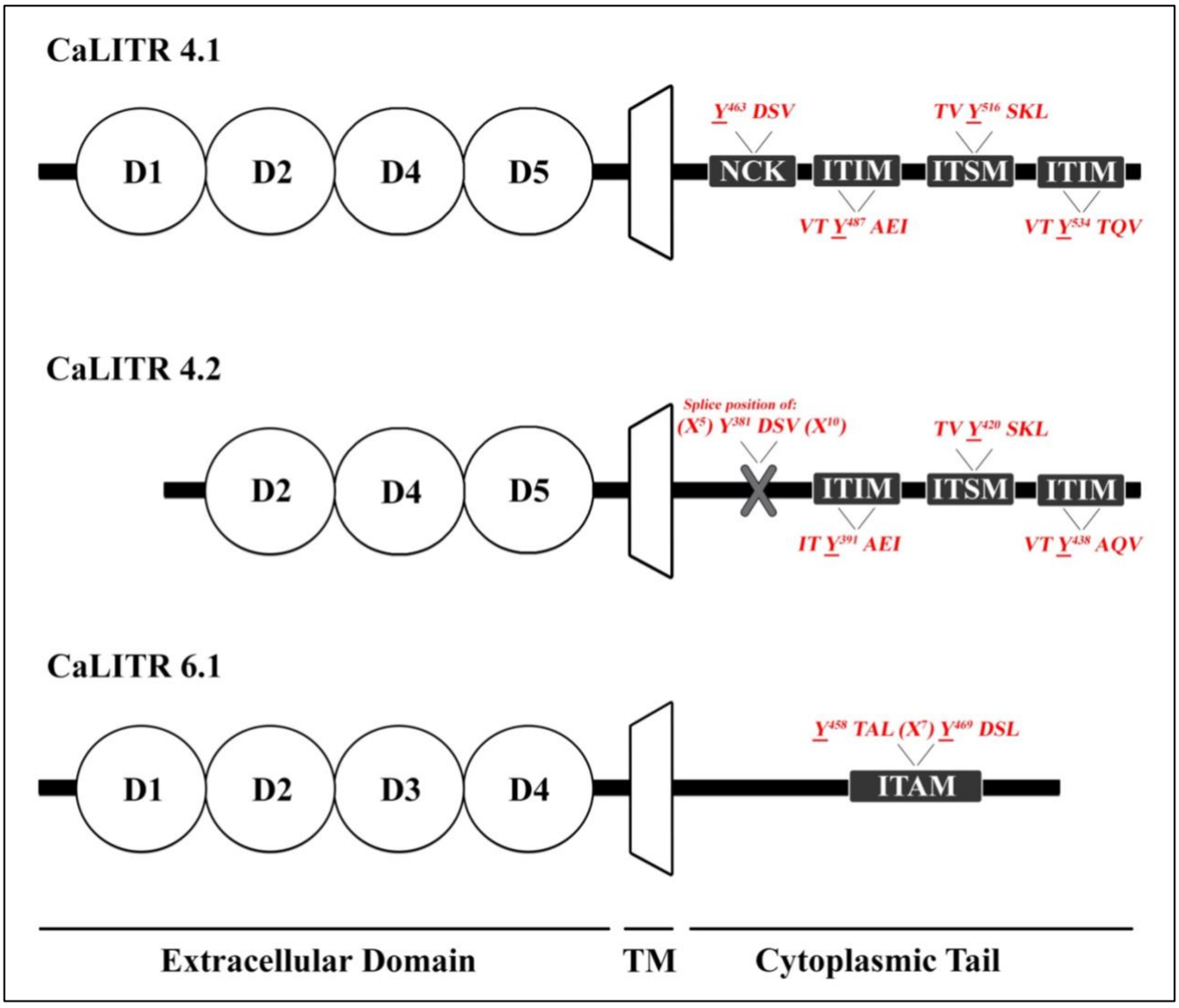
150
100
50
0
160
120
80
40
10^0 10^1 10^2 10^3 10^4 10^5 10^6

Figure 4.1. Expression profiles of native and chimeric (C)-CaLITRs transfected into AD293 cells. CaLITRs (*Carassius Auratus*) cDNA was amplified via PCR and sub-cloned into pDisplay expression vector using SmaI/PstI or SmaI/SalI digestion enzymes. Parental (non-transfected) AD293 cells were transfected with (**A**) No Vector (left), Empty pDisplay Vector (right), (**B**) native CaLITR 2.0, (**C**) native CaLITR 2.1, (**D**) native CaLITR 4.1 (left) and native CaLITR 6.1 (right), (**E**) chimeric CaLITR 2.0 and 2.1, and (**F**) chimeric CaLITR 6.1 and the positive control, IpLITR 2.6b. All transfected cell populations underwent antibiotic selection and were cloned after FACS sorting. Cell clones for each CaLITR construct were then primarily stained with mouse anti-HA mAb and secondarily stained with PE-conjugated goat-anti-mouse pAb. Samples were visualized for their LITR protein surface expression with the Attune NxT flow cytometer (ThermoFisher). Cell events were gated initially based on FSC-A versus SSC-A parameters to remove debris and large cellular clumps, then FSC-A was gated versus FSC-H to remove doublets. PE fluorescence intensity was analyzed as a histogram for cellular events where anti-HA stained cells (green) were compared to IgG_1 isotype control-stained cells (red). (**F**, right) IpLITR 2.6b was used as a positive control for HA-tagged protein on the surface of AD293 cells.

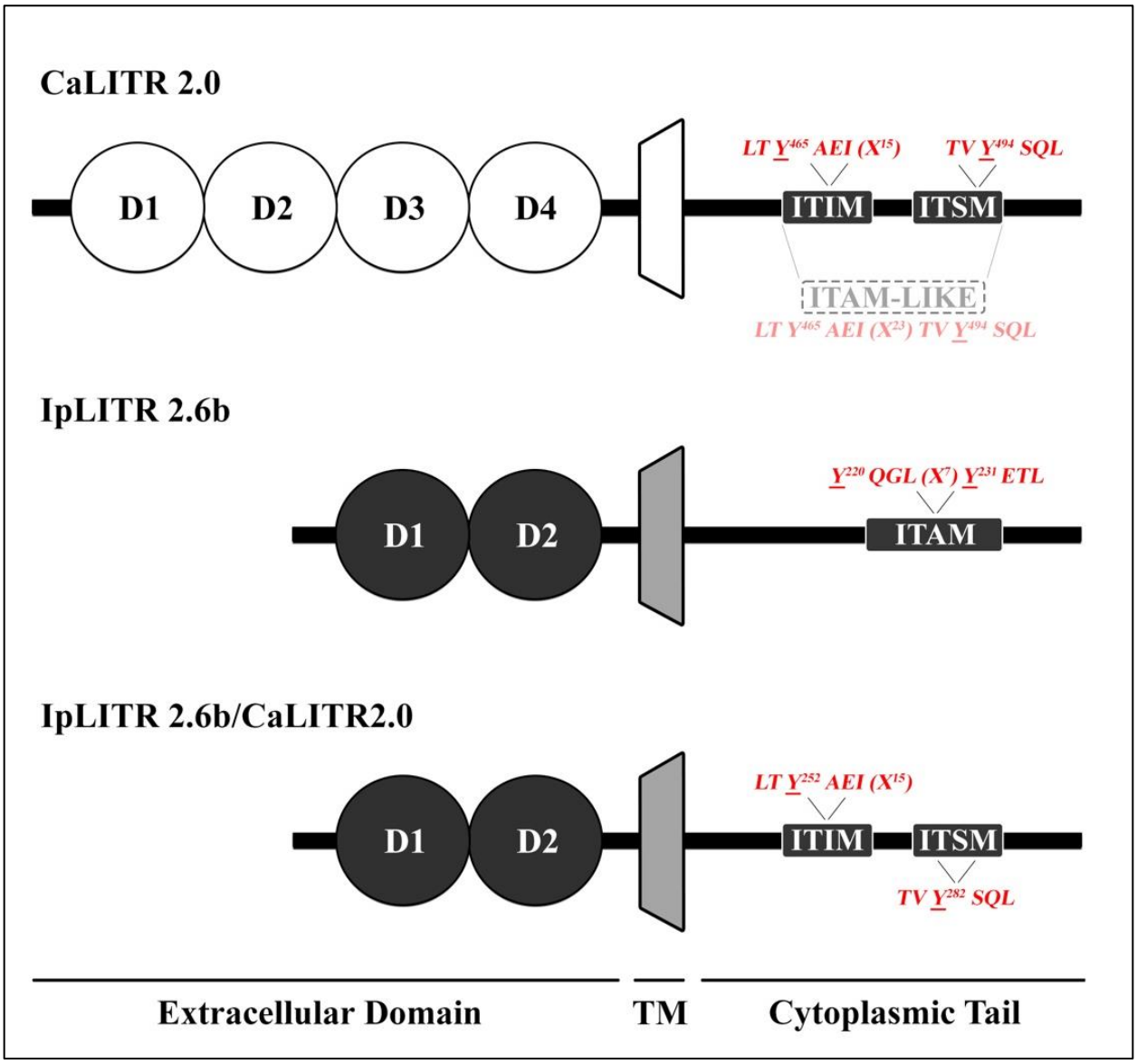
A.


CaLITR 2.0
D1
D2
D3
D4
LT Y465 AEI (X15)
TV Y494 SQL
ITIM
ITSM
ITAM-LIKE
LT Y465 AEI (X23) TV Y494 SQL
CaLITR 2.1
Splice position of:
(X8) LT Y465 AEI (X15)
D1
D2
D3
D4
ITSM
TV Y463 SQL

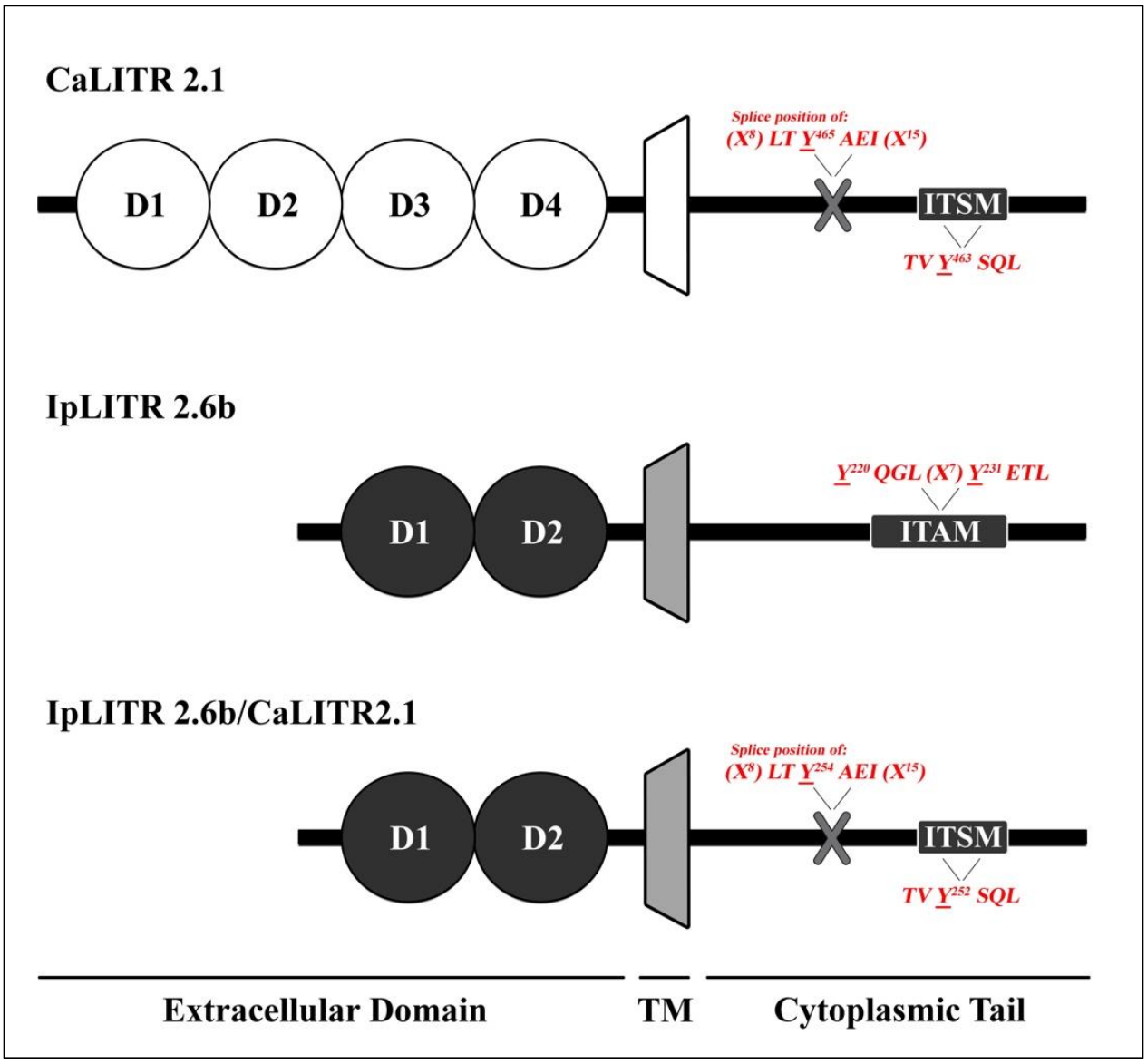
B.


CaLITR 4.1
D1
D2
D4
D5
Y463 DSV
TV Y516 SKL
NCK
ITIM
ITSM
ITIM
VT Y487 AEI
VT Y534 TQV
CaLITR 4.2
Splice position of:
(X5) Y381 DSV (X10)
TV Y420 SKL
D2
D4
D5
ITIM
ITSM
ITIM
IT Y391 AEI
VT Y438 AQV
CaLITR 6.1
Y458 TAL (X7) Y469 DSL
D1
D2
D3
D4
ITAM
Extracellular Domain
TM
Cytoplasmic Tail

C.


CaLITR 2.0
D1
D2
D3
D4
LT Y465 AEI (X15)
TV Y494 SQL
ITIM
ITSM
ITAM-LIKE
LT Y465 AEI (X23) TV Y494 SQL
IpLITR 2.6b
D1
D2
Y220 QGL (X7) Y231 ETL
ITAM
IpLITR 2.6b/CaLITR2.0
D1
D2
LT Y252 AEI (X15)
ITIM
ITSM
TV Y282 SQL
Extracellular Domain
TM
Cytoplasmic Tail

D.


CaLITR 2.1
Splice position of:
(X8) LT Y465 AEI (X15)
D1
D2
D3
D4
ITSM
TV Y463 SQL
IpLITR 2.6b
Y220 QGL (X7) Y231 ETL
D1
D2
ITAM
IpLITR 2.6b/CaLITR2.1
Splice position of:
(X8) LT Y254 AEI (X15)
D1
D2
ITSM
TV Y252 SQL
Extracellular Domain
TM
Cytoplasmic Tail

E.

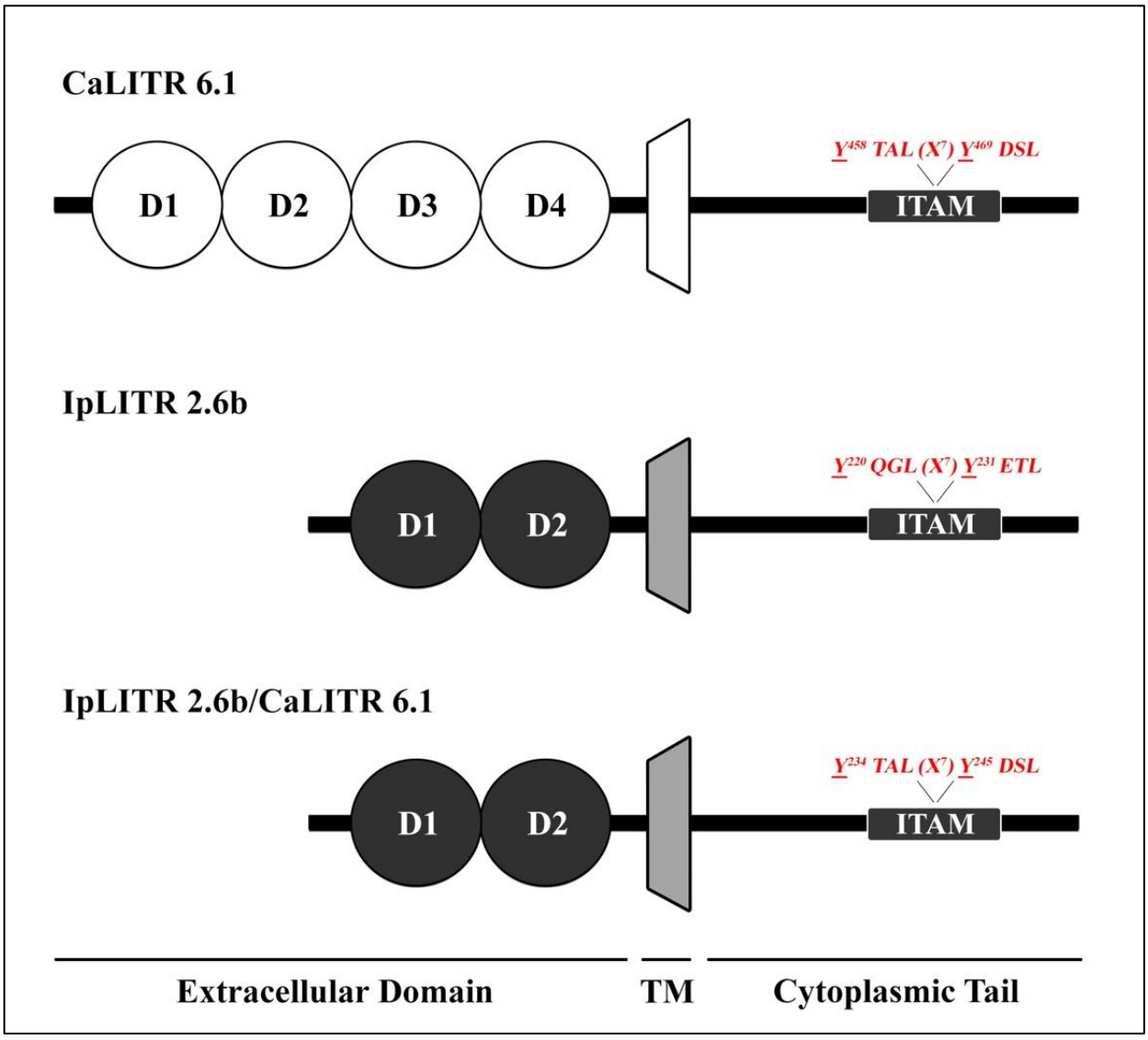
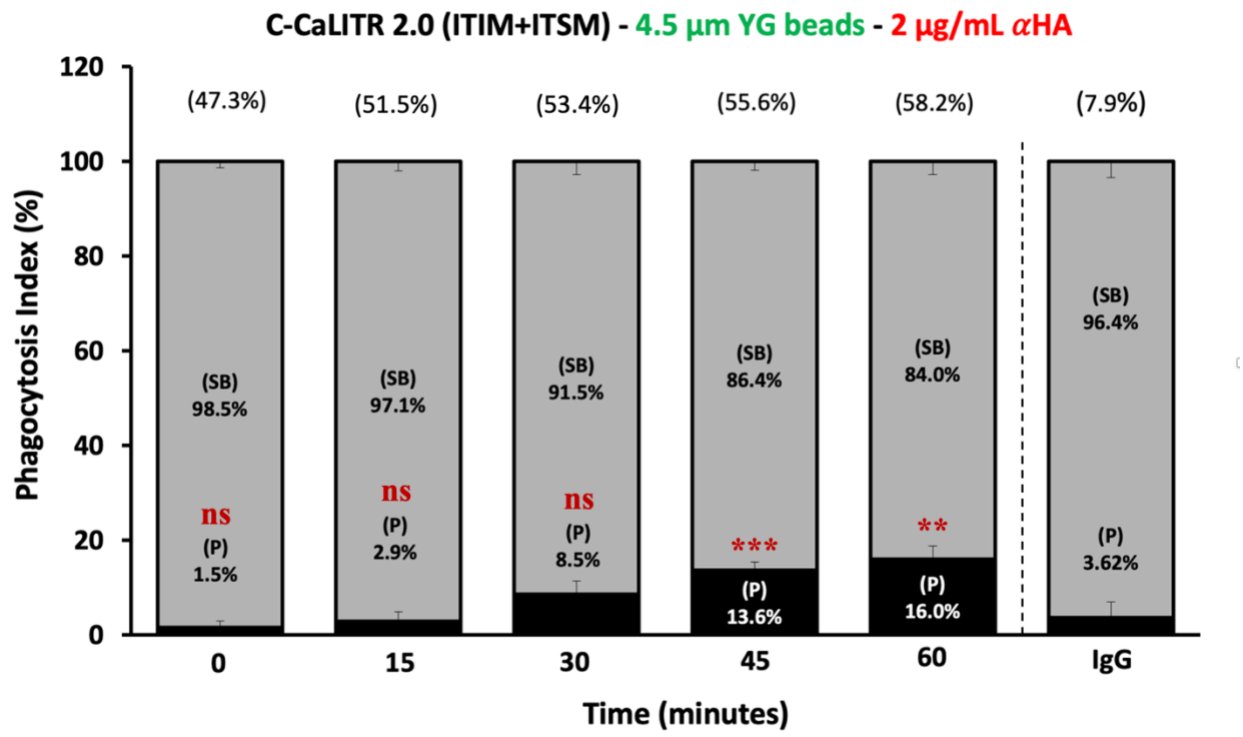
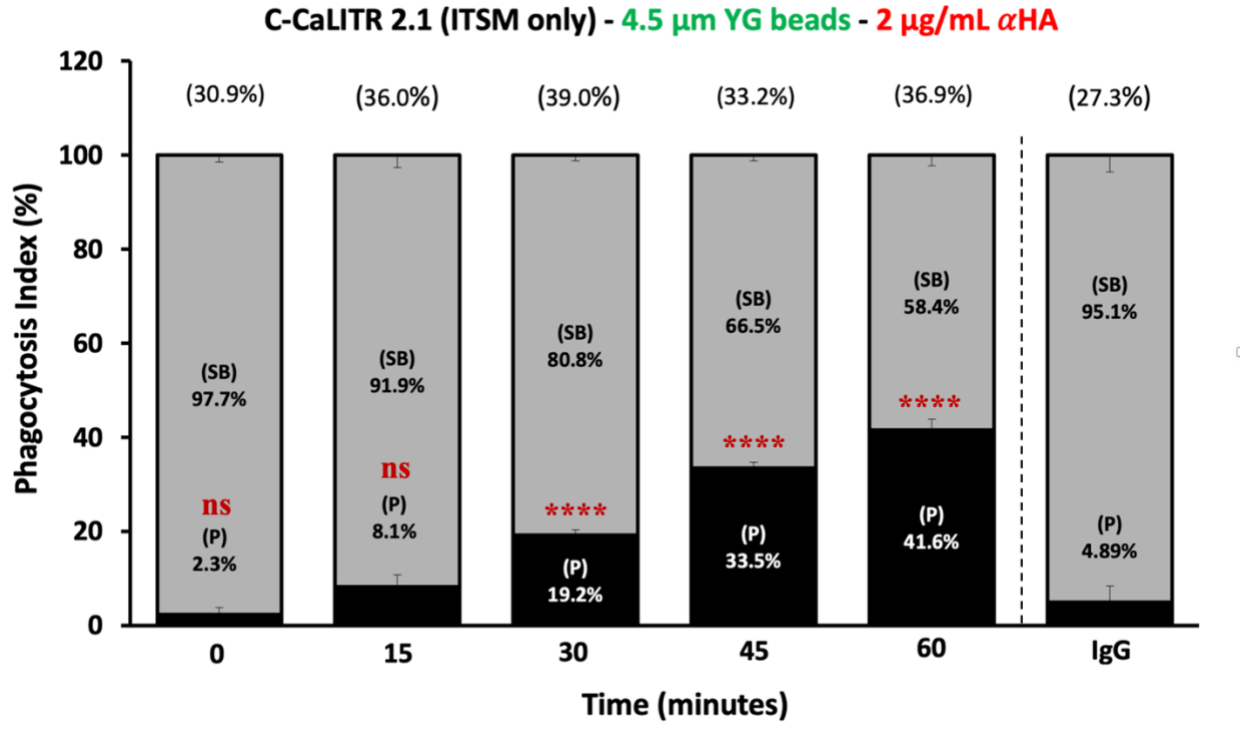

CaLITR 6.1
D1
D2
D3
D4
Y458 TAL (X7) Y469 DSL
ITAM
IpLITR 2.6b
D1
D2
Y220 QGL (X7) Y231 ETL
ITAM
IpLITR 2.6b/CaLITR 6.1
D1
D2
Y234 TAL (X7) Y245 DSL
ITAM
Extracellular Domain
TM
Cytoplasmic Tail

Figure 4.2. Schematic representations of native and chimeric (C)-CaLITR constructs. (**A**) Images depict protein receptors containing immunoglobulin domains (D1, D2, etc.), a transmembrane region, and a cytoplasmic tail (CYT) region. CaLITR 2.0 was initially thought to contain an immunoreceptor tyrosine-based activation (ITAM)-like motif. However, upon further examination, it was found that the first tyrosine region resembles that of an immunoreceptor tyrosine-based inhibition motif (ITIM), while the second tyrosine resembles that of an immunoreceptor tyrosine-based switch motif (ITIM). CaLITR 2.1 is a splice variant of 2.0, with a 29 amino acid deletion in the CYT, leaving only the ITSM motif. (**B**) CaLITR 4.1 contains an Nck recruitment motif, an ITIM, ITSM and ITIM motifs in tandem. While CaLITR 4.2 contains the same motifs as 4.1 except for the Nck recruitment motif due to CYT region deletion. CaLITR 6.1 contains an immunoreceptor tyrosine-based activation motif (ITAM). Chimeric CaLITR (**C**) 2.0, (**D**) 2.1, and (**E**) 6.1 were made by fusing the CYT region of the corresponding native CaLITR with the TM and extracellular domain of the positive transfection control, IpLITR 2.6b.

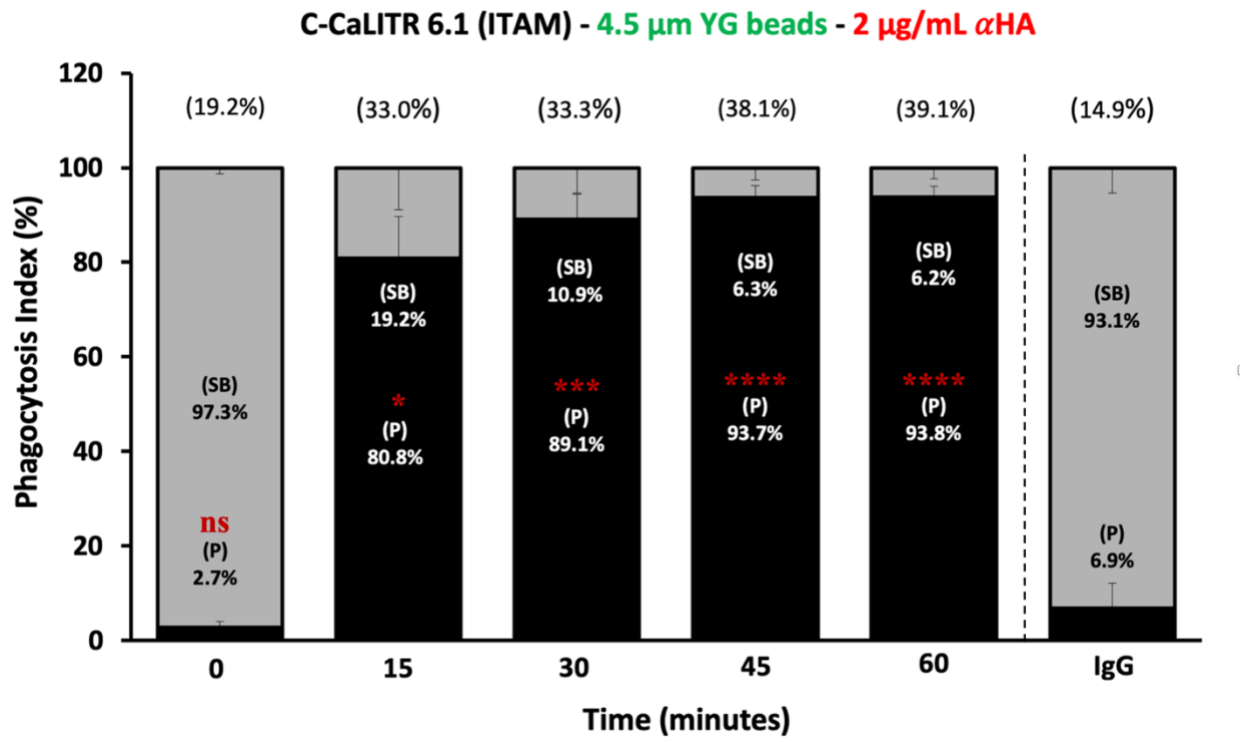
A.


C-CaLITR 2.0 (ITIM+ITSM) - 4.5 µm YG beads - 2 µg/mL αHA
Phagocytosis Index (%)
120
100
80
60
40
20
0
(47.3%)
(51.5%)
(53.4%)
(55.6%)
(58.2%)
(7.9%)
(SB) 98.5%
ns
(P) 1.5%
(SB) 97.1%
ns
(P) 2.9%
(SB) 91.5%
ns
(P) 8.5%
(SB) 86.4%
***
(P) 13.6%
(SB) 84.0%
**
(P) 16.0%
(SB) 96.4%
(P) 3.62%
0
15
30
45
60
IgG
Time (minutes)

B.


C-CaLITR 2.1 (ITSM only) - 4.5 µm YG beads - 2 µg/mL αHA
Phagocytosis Index (%)
120
100
80
60
40
20
0
(30.9%)
(36.0%)
(39.0%)
(33.2%)
(36.9%)
(27.3%)
(SB) 97.7%
ns
(P) 2.3%
(SB) 91.9%
ns
(P) 8.1%
(SB) 80.8%
****
(P) 19.2%
(SB) 66.5%
****
(P) 33.5%
(SB) 58.4%
****
(P) 41.6%
(SB) 95.1%
(P) 4.89%
0
15
30
45
60
IgG
Time (minutes)

C.


C-CaLITR 6.1 (ITAM) - 4.5 µm YG beads - 2 µg/mL αHA
Phagocytosis Index (%)
120
100
80
60
40
20
0
(19.2%)
(33.0%)
(33.3%)
(38.1%)
(39.1%)
(14.9%)
(SB)
97.3%
ns
(P)
2.7%
(SB)
19.2%
*
(P)
80.8%
(SB)
10.9%
***
(P)
89.1%
(SB)
6.3%
****
(P)
93.7%
(SB)
6.2%
****
(P)
93.8%
(SB)
93.1%
(P)
6.9%
0
15
30
45
60
IgG
Time (minutes)

D.

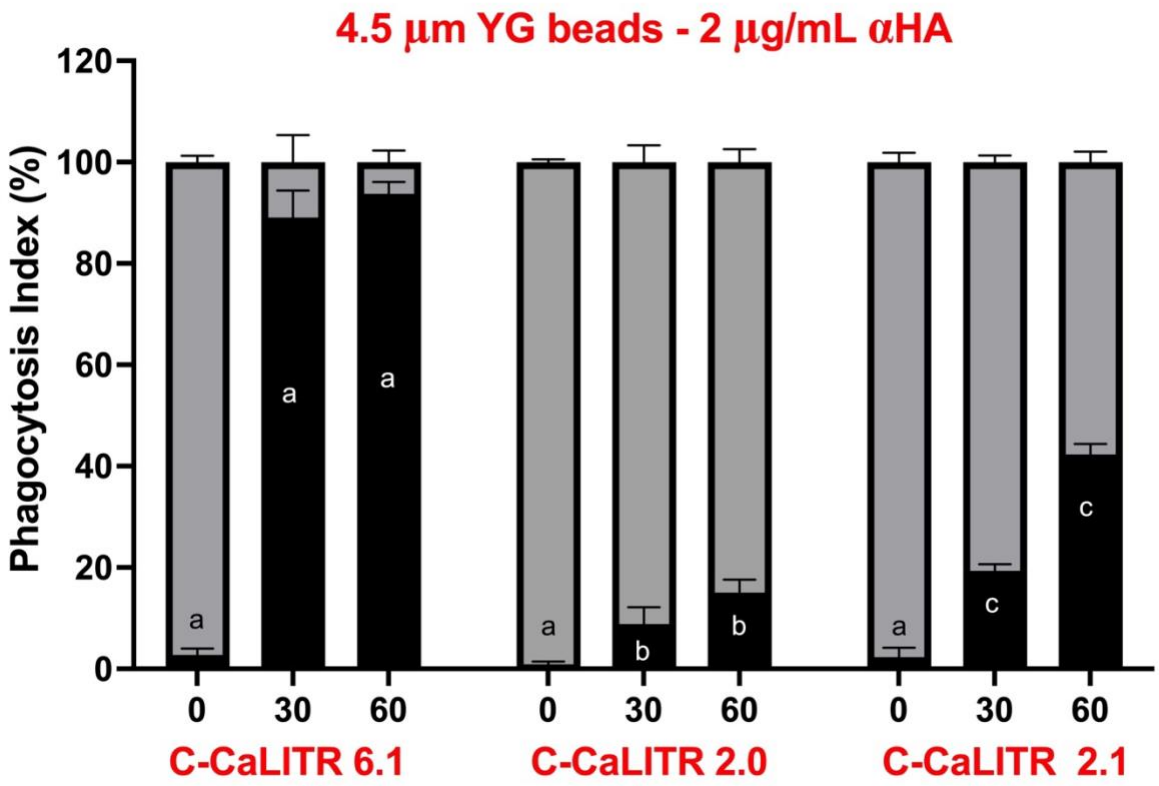
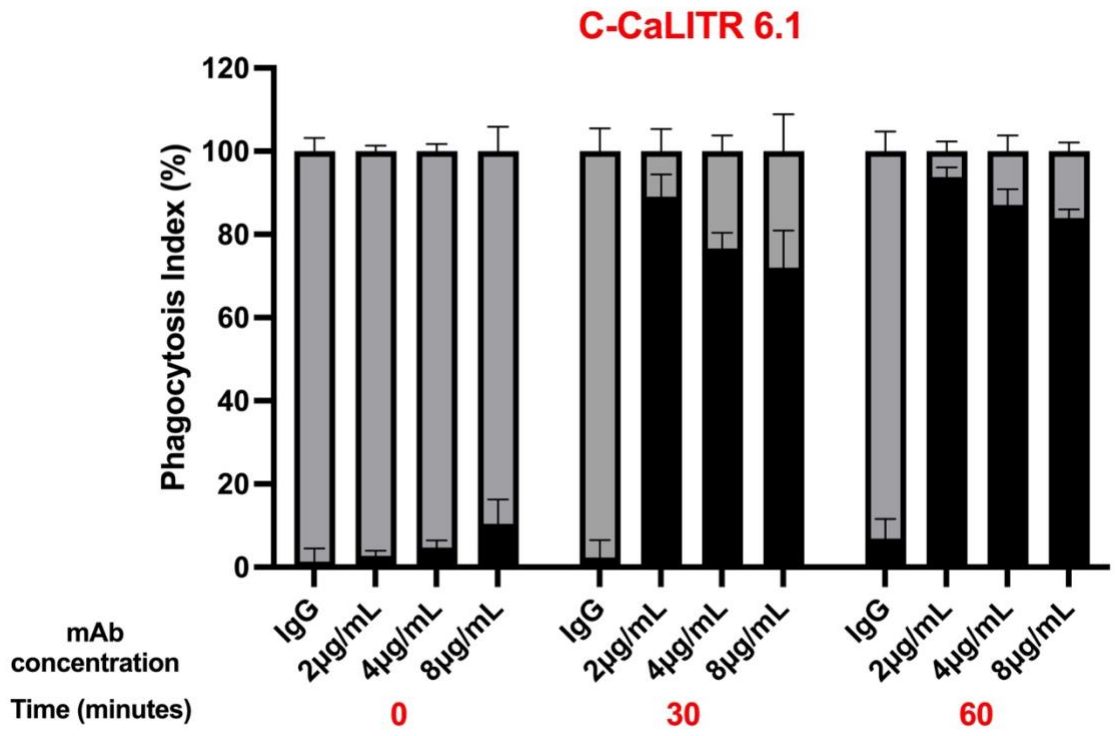
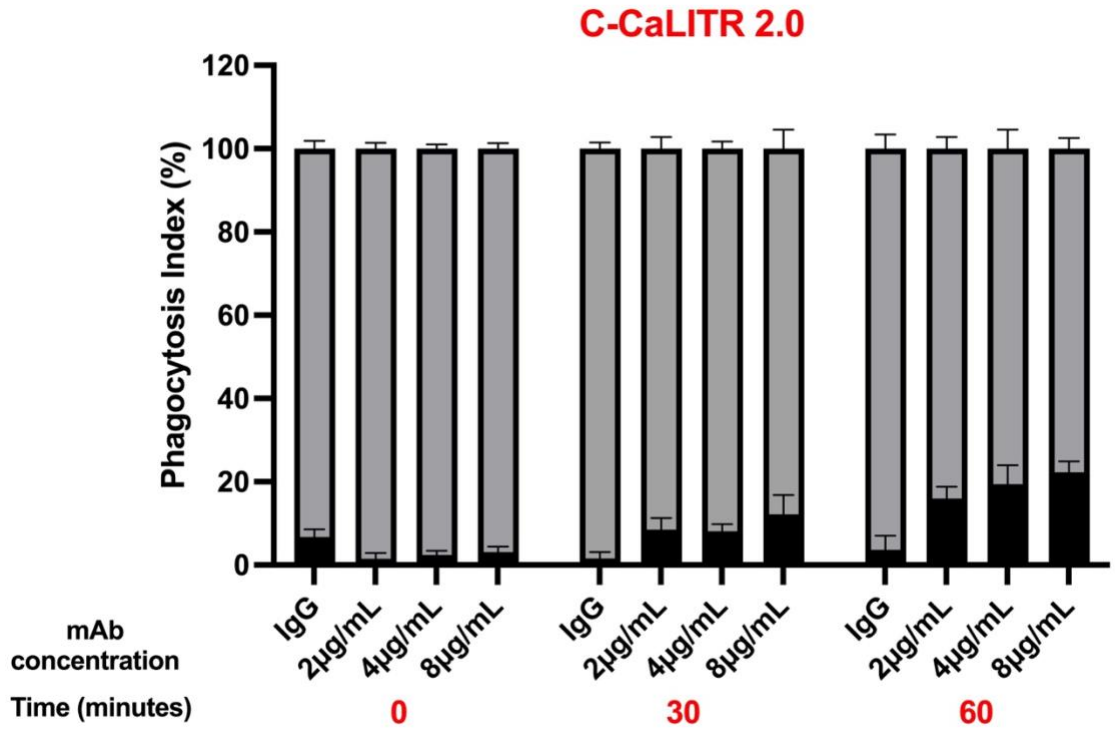

4.5 µm YG beads - 2 µg/mL αHA
Phagocytosis Index (%)
120
100
80
60
40
20
0
a
a
a
a
b
b
a
c
c
0
30
60
0
30
60
0
30
60
C-CaLITR 6.1
C-CaLITR 2.0
C-CaLITR 2.1

Figure 4.3. Phagocytosis assays reveal differences between C-CaLITR-expressing AD293 cells through imaging flow cytometry. 3 x 10^5 AD293 cells expressing C-CaLITR (**A**) 2.0, (**B**) 2.1, and (**C**) 6.1. were incubated with 4.5μm YG beads (9 x 10^5), coated in protein G, followed by opsonization with 2 μg/mL αHA mAb, for 60 minutes at 37°C. Samples were then counter-stained and subsequently analyzed using the ImageStream X Mark II. Events were classified as phagocytic (P; black bars) with at least 1 bead being internalized or surfaced bound (SB; grey bars) with only surface attached beads. Samples were normalized, and the % values were calculated as # of surface-bound events or # of phagocytic events / # of all bead-associated events. Each bar represents the mean ± SEM of total cells associated with beads from 3 independent experiments. The bracketed % value above each bar represents the proportion of cells associated with beads. Statistical (temporal) comparisons for each construct were analyzed using the Brown-Forsythe and Welch ANOVA test, (*) representing statistical significance ($p \leq 0.05$) between phagocytosis (%) means at each time point vs IgG isotype. (**D**) Shows summary graph for all three constructs using time points 0, 30 and 60 minutes. Sample data groups were analyzed using a one-way ANOVA and Tukey test (Prism 10, GraphPad, La Jolla, CA, USA). Bars containing different letters (*a, b, or c*) represent statistical significance ($p \leq 0.05$) between phagocytosis (%) means of different constructs at the same time point.

A.


C-CaLITR 6.1
Phagocytosis Index (%)
120
100
80
60
40
20
0
IgG
2µg/mL
4µg/mL
8µg/mL
IgG
2µg/mL
4µg/mL
8µg/mL
IgG
2µg/mL
4µg/mL
8µg/mL
mAb concentration
Time (minutes)
0
30
60

B.


C-CaLITR 2.0
Phagocytosis Index (%)
120
100
80
60
40
20
0
IgG
2µg/mL
4µg/mL
8µg/mL
mAb concentration
Time (minutes)
0
30
60

C.

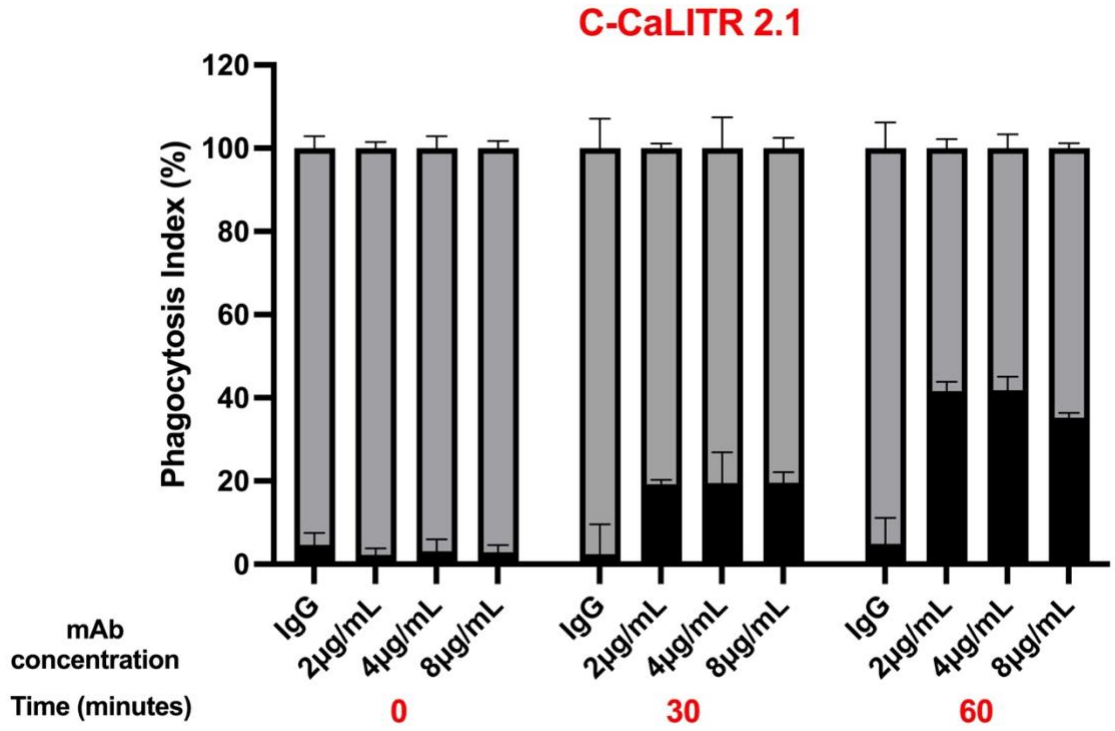
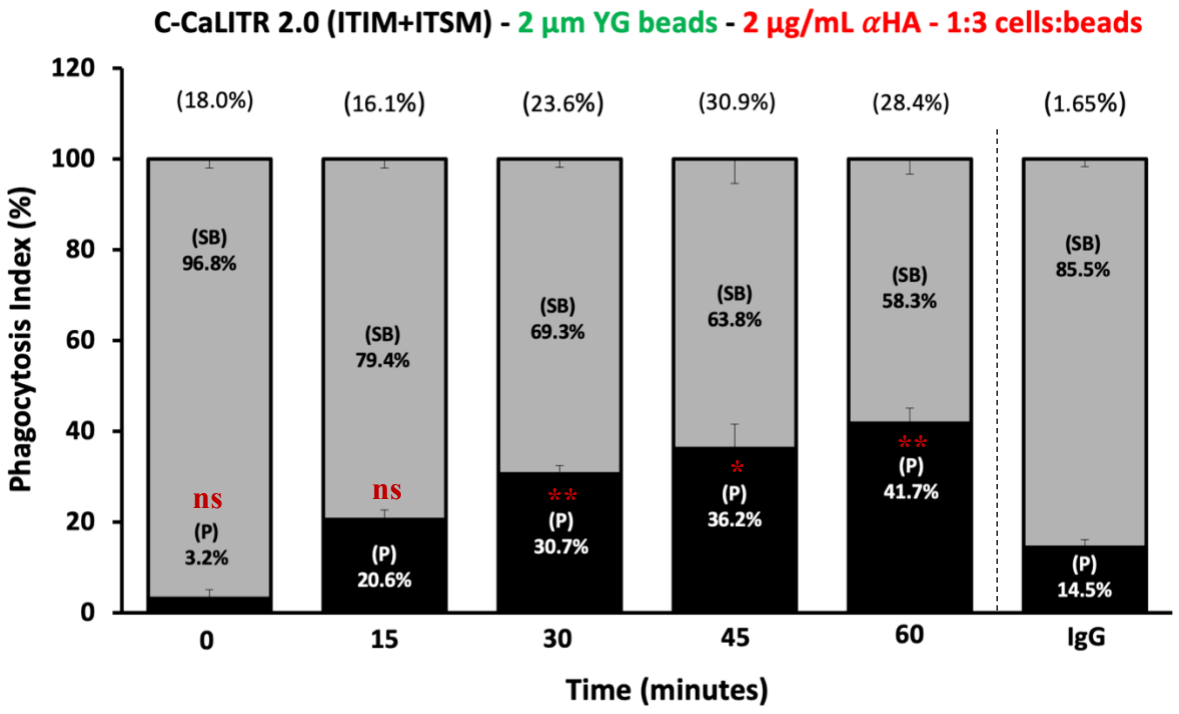
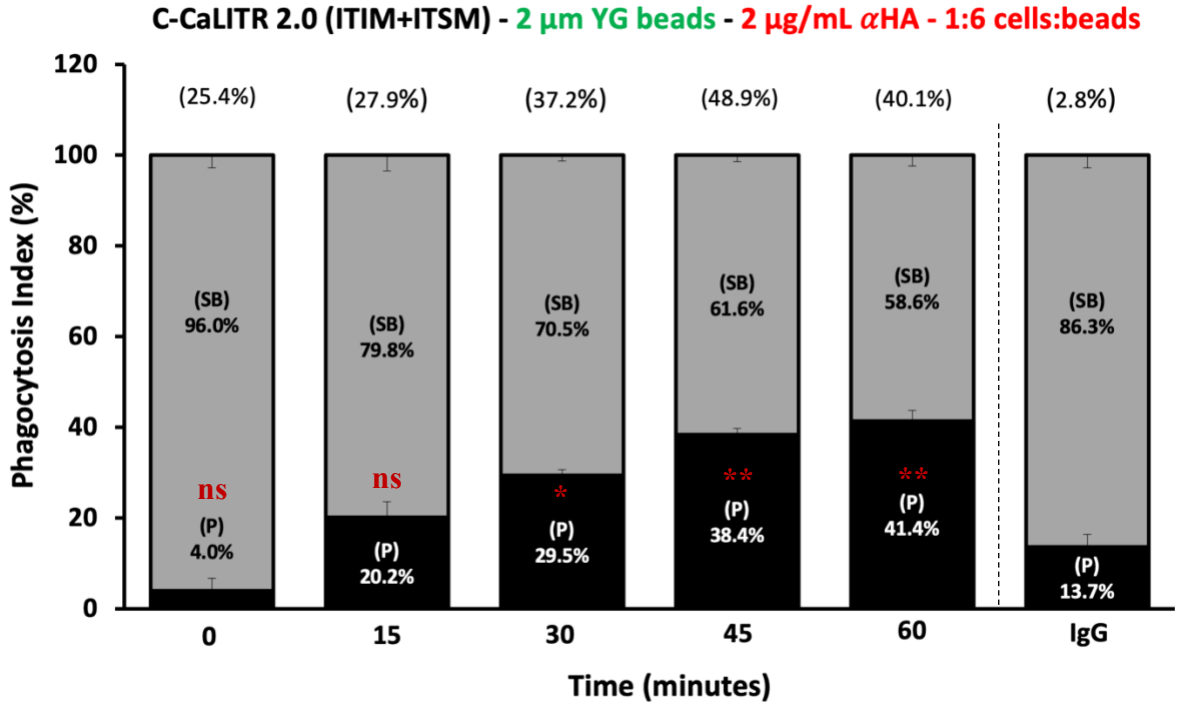

C-CaLITR 2.1
Phagocytosis Index (%)
120
100
80
60
40
20
0
IgG
2µg/mL
4µg/mL
8µg/mL
mAb concentration
Time (minutes)
0
30
60

Figure 4.4. Increasing concentrations of αHA mAb used to opsonize YG-bead targets reveal consistent phagocytic activity. 3 x 10^5 AD293 cells expressing: (**A**) C-CaLITR 6.1, (B) C-CaLITR 2.0, and (**C**) C-CaLITR 2.1, were incubated with 4.5μm YG beads (9 x 10^5), coated in protein G, followed by opsonization with either: (left to right columns) 2 μg/mL IgG Isotype control, 2 μg/mL, 4 μg/mL, and 8 μg/mL of αHA mAb for 60 minutes at 37°C. Samples were then counter-stained and subsequently analyzed using the ImageStream X Mark II. Events were classified as phagocytic (P, black bars) with at least 1 bead being internalized or surfaced bound (SB, grey bars) with only surface attached beads. Samples were normalized, and the % values were calculated as # of surface-bound events or # of phagocytic events / # of all bead-associated events. Each bar represents the mean ± SEM of total cells associated with beads from 3 independent experiments.

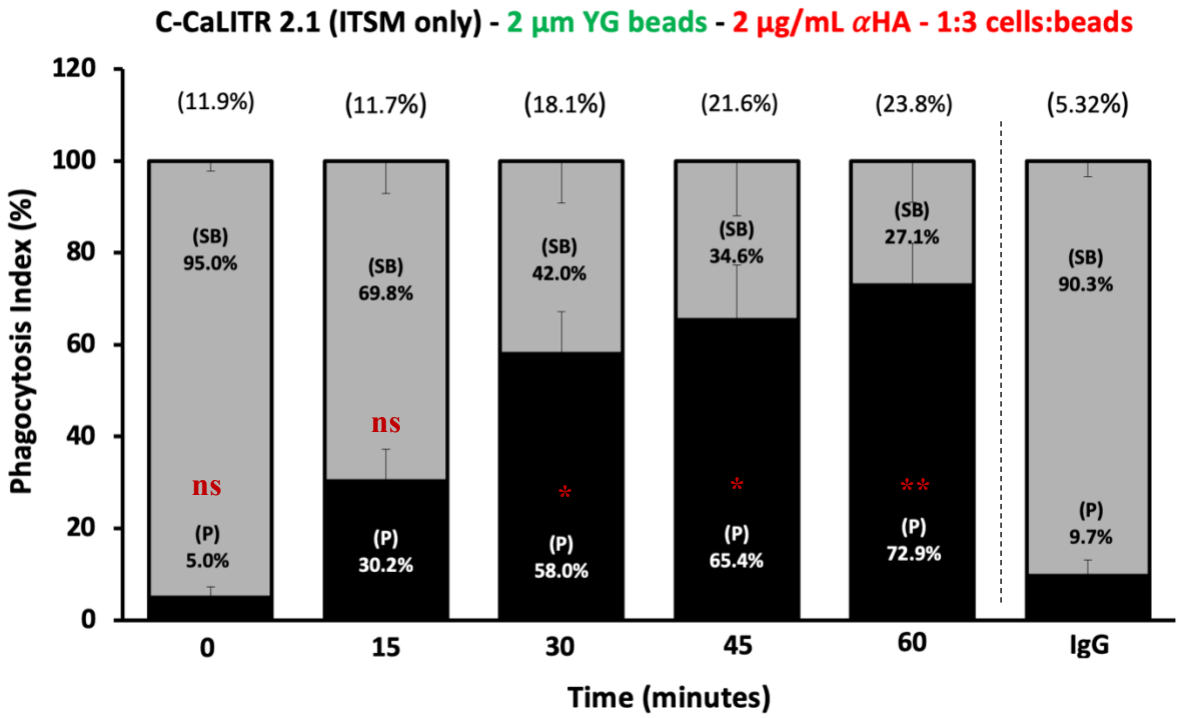
A.


C-CaLITR 2.0 (ITIM+ITSM) - 2 µm YG beads - 2 µg/mL αHA - 1:3 cells:beads
Phagocytosis Index (%)
120
100
80
60
40
20
0
(18.0%)
(16.1%)
(23.6%)
(30.9%)
(28.4%)
(1.65%)
(SB) 96.8%
ns
(P) 3.2%
(SB) 79.4%
ns
(P) 20.6%
(SB) 69.3%
**
(P) 30.7%
(SB) 63.8%
*
(P) 36.2%
(SB) 58.3%
**
(P) 41.7%
(SB) 85.5%
(P) 14.5%
0
15
30
45
60
IgG
Time (minutes)

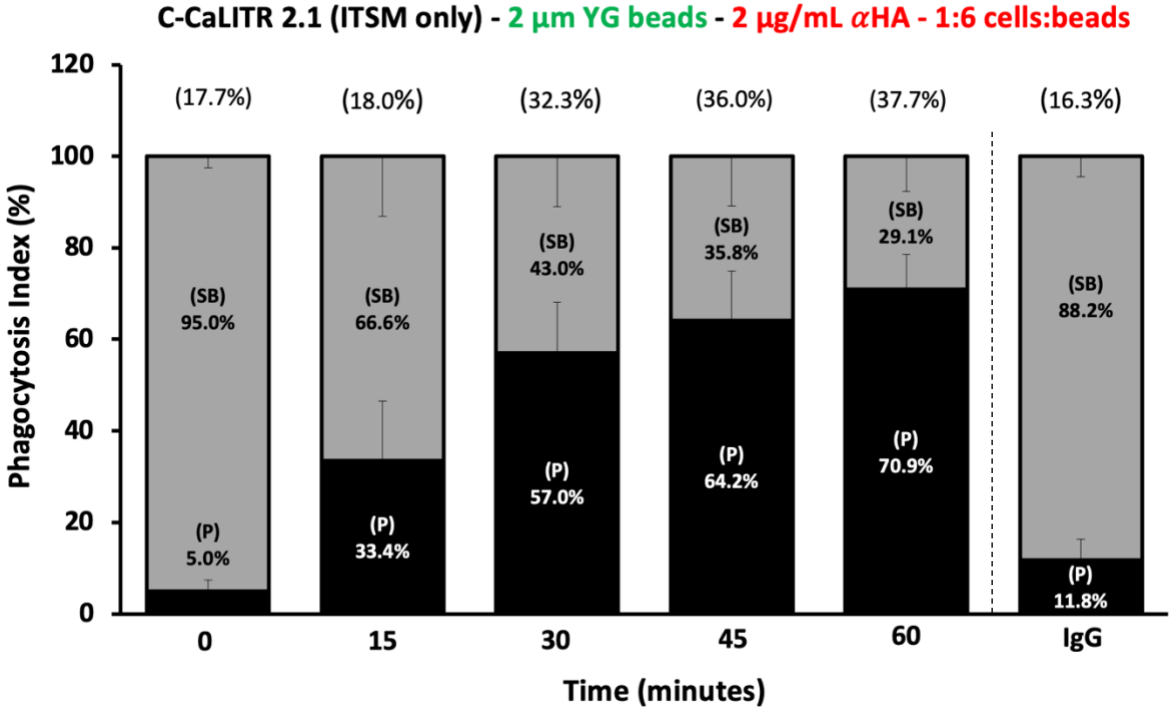
B.


C-CaLITR 2.0 (ITIM+ITSM) - 2 µm YG beads - 2 µg/mL αHA - 1:6 cells:beads
Phagocytosis Index (%)
120
100
80
60
40
20
0
(25.4%)
(27.9%)
(37.2%)
(48.9%)
(40.1%)
(2.8%)
(SB) 96.0%
ns
(P) 4.0%
(SB) 79.8%
ns
(P) 20.2%
(SB) 70.5%
*
(P) 29.5%
(SB) 61.6%
**
(P) 38.4%
(SB) 58.6%
**
(P) 41.4%
(SB) 86.3%
(P) 13.7%
0
15
30
45
60
IgG
Time (minutes)

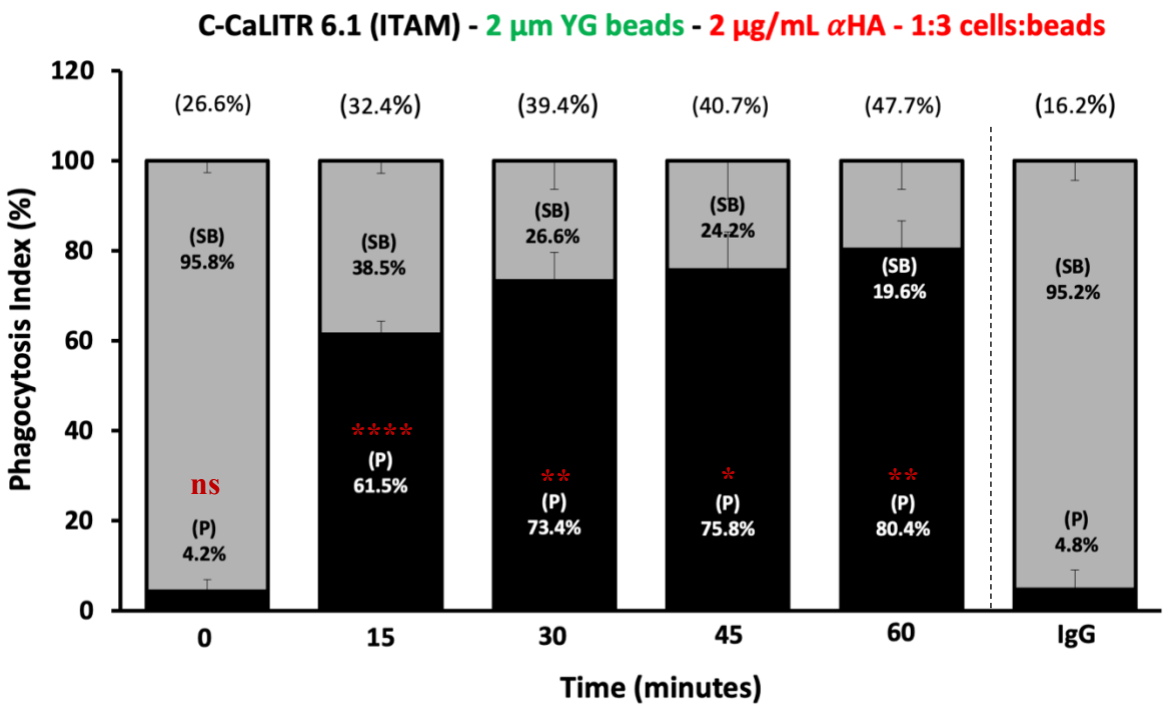
C.


C-CaLITR 2.1 (ITSM only) - 2 µm YG beads - 2 µg/mL αHA - 1:3 cells:beads
Phagocytosis Index (%)
120
100
80
60
40
20
0
(11.9%)
(11.7%)
(18.1%)
(21.6%)
(23.8%)
(5.32%)
(SB) 95.0%
(SB) 69.8%
(SB) 42.0%
(SB) 34.6%
(SB) 27.1%
(SB) 90.3%
ns
ns
*
*
**
(P) 5.0%
(P) 30.2%
(P) 58.0%
(P) 65.4%
(P) 72.9%
(P) 9.7%
0
15
30
45
60
IgG
Time (minutes)

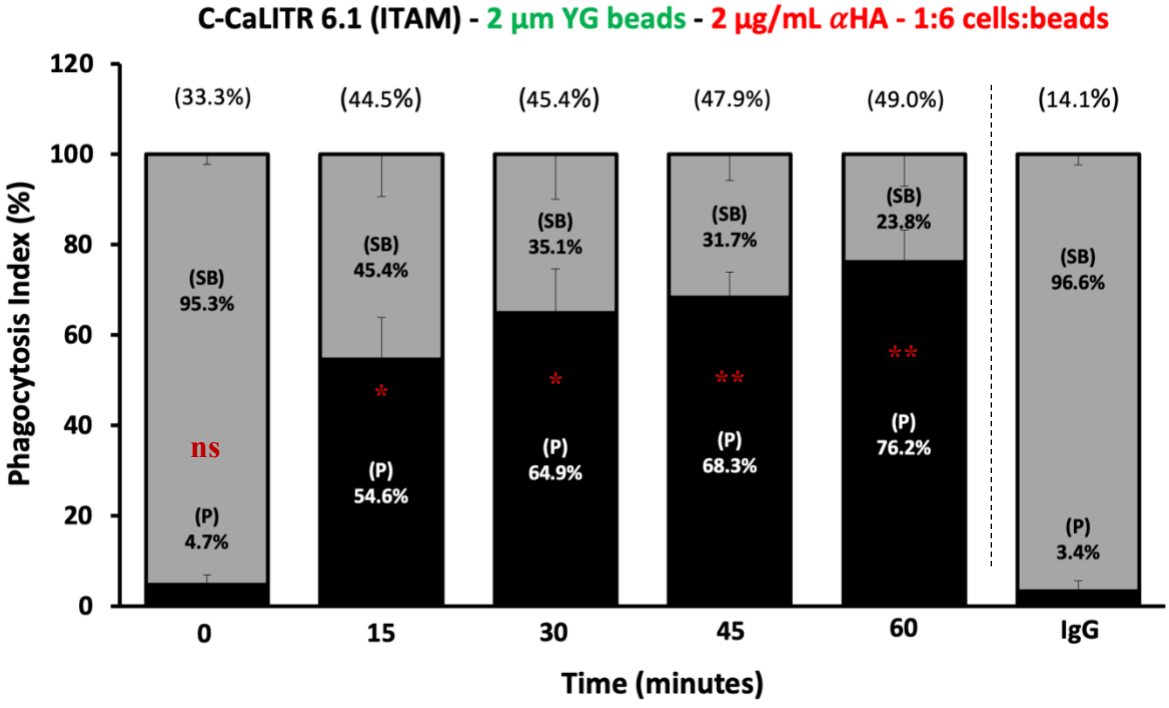
D.


C-CaLITR 2.1 (ITSM only) - 2 µm YG beads - 2 µg/mL αHA - 1:6 cells:beads
Phagocytosis Index (%)
120
100
80
60
40
20
0
(17.7%)
(18.0%)
(32.3%)
(36.0%)
(37.7%)
(16.3%)
(SB) 95.0%
(SB) 66.6%
(SB) 43.0%
(SB) 35.8%
(SB) 29.1%
(SB) 88.2%
(P) 5.0%
(P) 33.4%
(P) 57.0%
(P) 64.2%
(P) 70.9%
(P) 11.8%
0
15
30
45
60
IgG
Time (minutes)

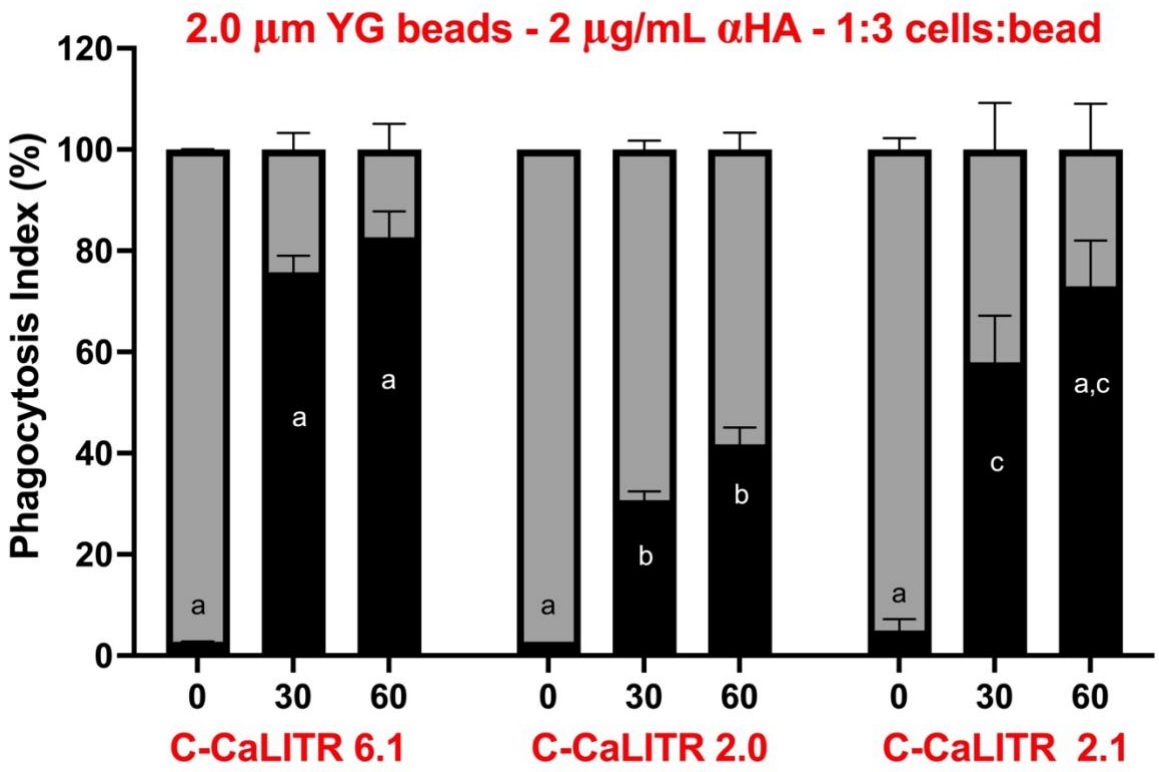
E.


C-CaLITR 6.1 (ITAM) - 2 µm YG beads - 2 µg/mL αHA - 1:3 cells:beads
Phagocytosis Index (%)
120
100
80
60
40
20
0
(26.6%)
(32.4%)
(39.4%)
(40.7%)
(47.7%)
(16.2%)
(SB) 95.8%
ns
(P) 4.2%
(SB) 38.5%
****
(P) 61.5%
(SB) 26.6%
**
(P) 73.4%
(SB) 24.2%
*
(P) 75.8%
(SB) 19.6%
**
(P) 80.4%
(SB) 95.2%
(P) 4.8%
0
15
30
45
60
IgG
Time (minutes)

F.


C-CaLITR 6.1 (ITAM) - 2 µm YG beads - 2 µg/mL αHA - 1:6 cells:beads
Phagocytosis Index (%)
120
100
80
60
40
20
0
(33.3%)
(44.5%)
(45.4%)
(47.9%)
(49.0%)
(14.1%)
(SB) 95.3%
ns
(P) 4.7%
(SB) 45.4%
*
(P) 54.6%
(SB) 35.1%
*
(P) 64.9%
(SB) 31.7%
**
(P) 68.3%
(SB) 23.8%
**
(P) 76.2%
(SB) 96.6%
(P) 3.4%
0
15
30
45
60
IgG
Time (minutes)

G.


2.0 µm YG beads - 2 µg/mL αHA - 1:3 cells:bead
Phagocytosis Index (%)
120
100
80
60
40
20
0
a
a
a
a
b
b
a
c
a,c
0
30
60
C-CaLITR 6.1
C-CaLITR 2.0
C-CaLITR 2.1

H.

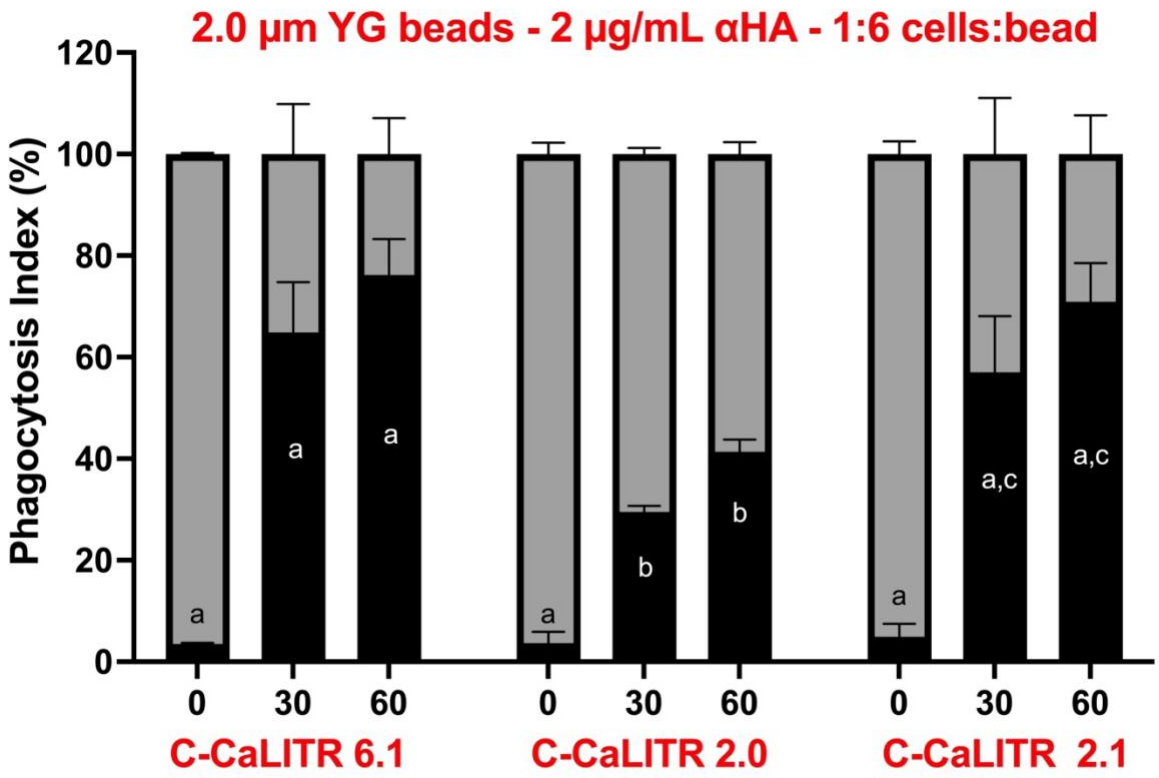
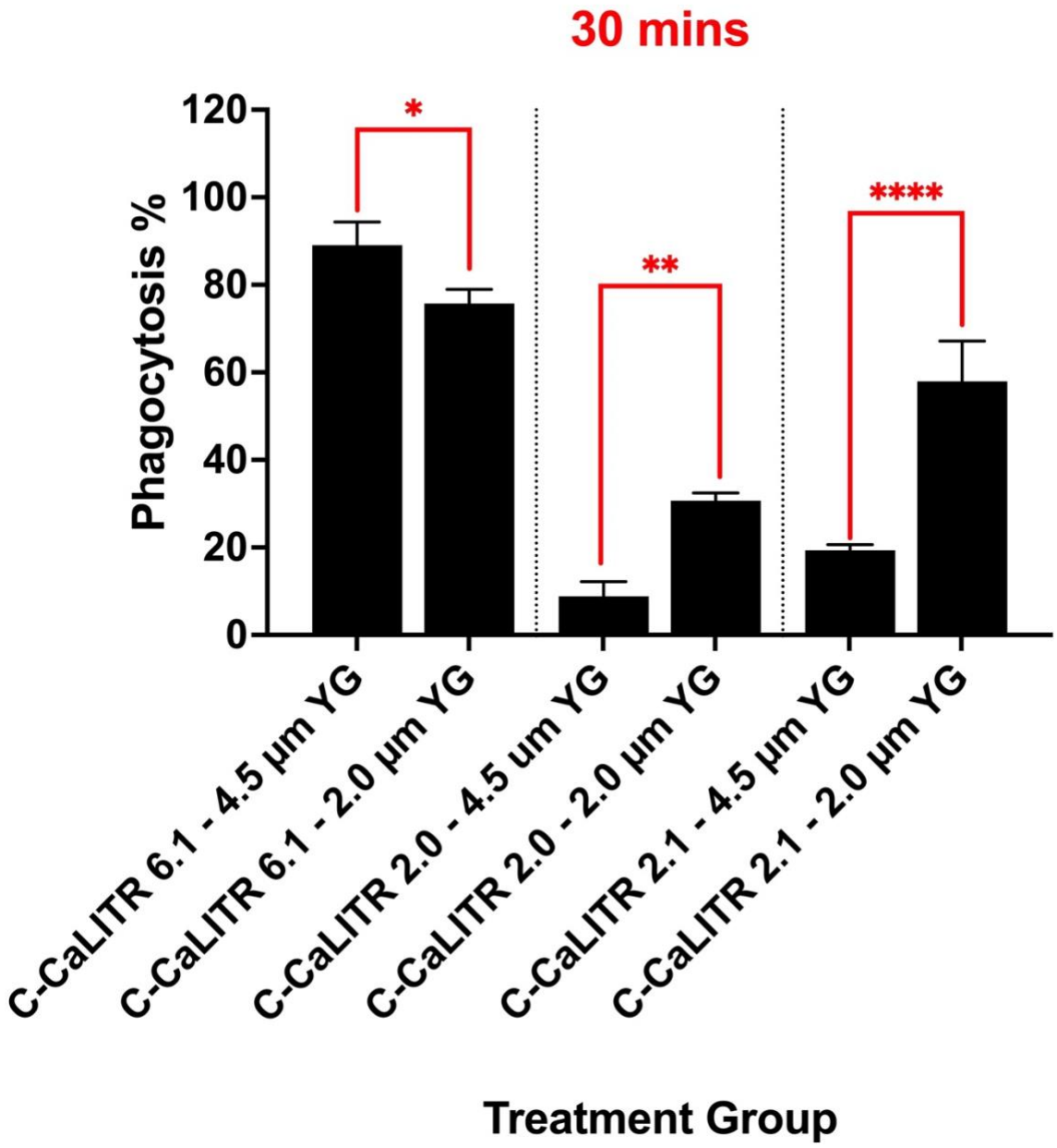

2.0 µm YG beads - 2 µg/mL αHA - 1:6 cells:bead
Phagocytosis Index (%)
120
100
80
60
40
20
0
a
a
a
a
b
b
a
a,c
a,c
0
30
60
0
30
60
0
30
60
C-CaLITR 6.1
C-CaLITR 2.0
C-CaLITR 2.1

Figure 4.5. Phagocytosis assays of smaller, 2.0 µm, YG-bead targets reveal subtle differences compared to 4.5 µm YG beads. 3 x 10^5 AD293 cells expressing C-CaLITR (**A, B**) 2.0, (**C, D**) 2.1, and (**E, F**) 6.1. were incubated with 2.0µm YG beads (9 x 10^5), coated in protein G, followed by opsonization with 2 µg/mL αHA mAb for 60 minutes at 37°C. Samples were then counter-stained and subsequently analyzed using the ImageStream X Mark II. Events were classified as phagocytic (P; black bars) with at least 1 bead being internalized or surfaced bound (S; grey bars) with only surface attached beads. Samples were normalized, and the % values were calculated as # of surface-bound events or # of phagocytic events / # of all bead-associated events. Each bar represents the mean ± SEM of total cells associated with beads from 3 independent experiments. The bracketed % value above each bar represents the proportion of cells associated with beads. Statistical (temporal) comparisons for each construct were analyzed using the Brown-Forsythe and Welch ANOVA test, (*) representing statistical significance ($p \leq 0.05$) between phagocytosis (%) means at each time point vs IgG isotype. Summary graphs that include time points 0, 30, and 60 minutes for all chimeric constructs are shown for two sets of experiments: (G) 1:3 cells to beads ratio and (**F**) for 1:6 cells to beads ratio. Sample data groups were analyzed using a one-way ANOVA and Tukey test (Prism 10, GraphPad, La Jolla, CA, USA). Bars containing different letters (*a, b,* or *c*) represent statistical significance ($p \leq 0.05$) between phagocytosis (%) means of different constructs at the same time point.

A.


30 mins
Phagocytosis %
120
100
80
60
40
20
0
*
**
****
C-CaLITR 6.1 - 4.5 µm YG
C-CaLITR 6.1 - 2.0 µm YG
C-CaLITR 2.0 - 4.5 um YG
C-CaLITR 2.0 - 2.0 µm YG
C-CaLITR 2.1 - 4.5 µm YG
C-CaLITR 2.1 - 2.0 µm YG
Treatment Group

B.

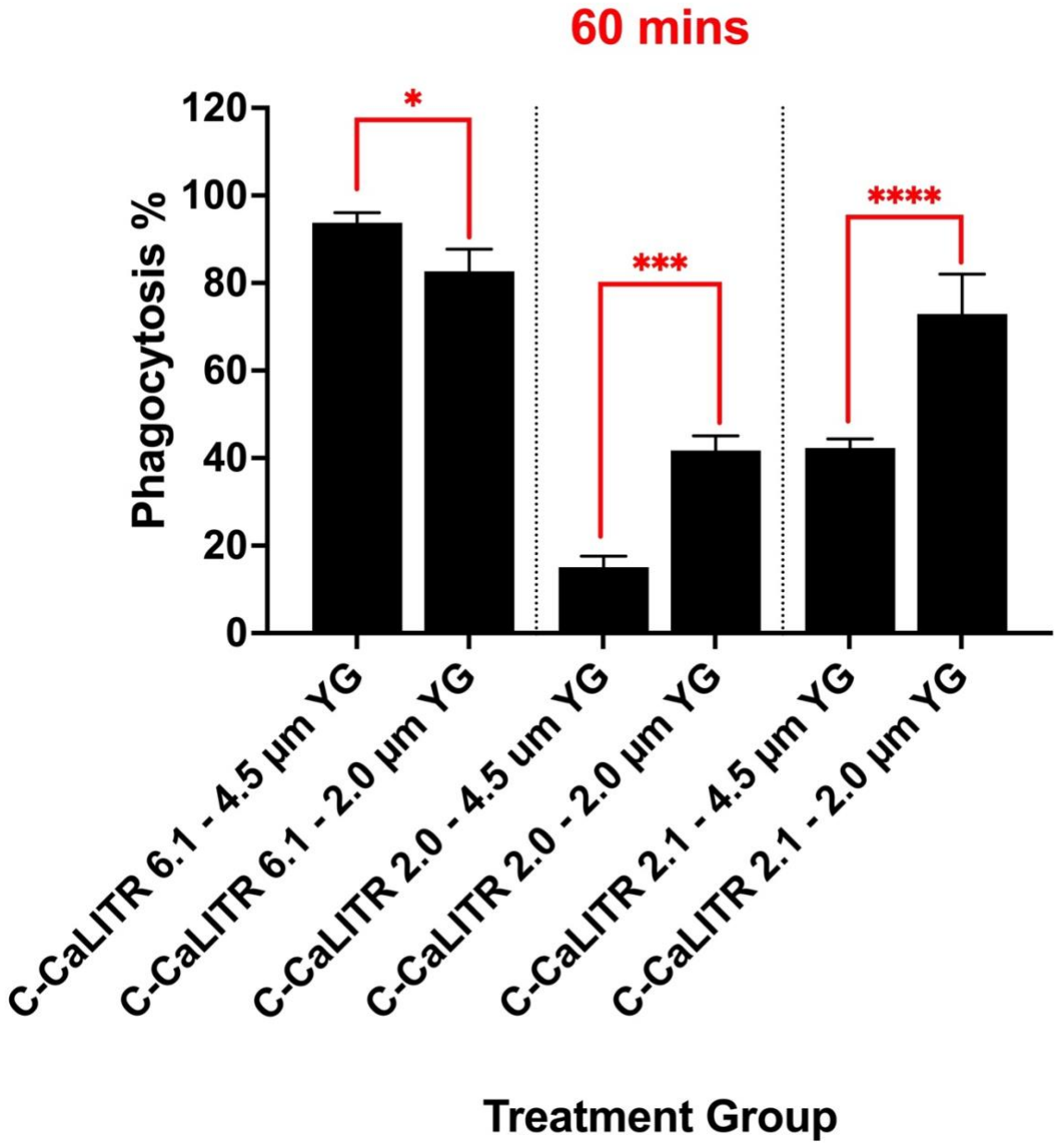

60 mins
Phagocytosis %
120
100
80
60
40
20
0
*
***
****
C-CaLITR 6.1 - 4.5 µm YG
C-CaLITR 6.1 - 2.0 µm YG
C-CaLITR 2.0 - 4.5 um YG
C-CaLITR 2.0 - 2.0 µm YG
C-CaLITR 2.1 - 4.5 µm YG
C-CaLITR 2.1 - 2.0 µm YG
Treatment Group

Figure 4.6. Summary graph of the Phagocytosis Assays by C-CaLITR constructs when stimulated with either 4.5 or 2.0 µm YG-bead targets. 3 x 10^5 AD293 cells expressing C-CaLITR 2.0, C-CaLITR 2.1, and C-CaLITR 6.1 were incubated with either 4.5 or 2.0 µm YG beads (9 x 10^5), coated in protein G, followed by opsonization with 2 µg/mL αHA mAb for (**A**) 30 minutes, and (**B**) 60 minutes at 37°C. Samples were then counter-stained and subsequently analyzed using the ImageStream X Mark II. Phagocytosis % resembles the percentage of cells with at least 1 internalized YG bead out of all events of cell-bead association. Each bar represents the mean ± SEM of total cells associated with beads from 3 independent experiments. Sample data groups were analyzed using a one-way ANOVA and Tukey test for multiple comparisons (Prism 10, GraphPad, La Jolla, CA, USA).

CHAPTER V

PHARMACOLOGICAL EXAMINATION OF CALITR-MEDIATED PHAGOCYTOSIS

5.1 Introduction

In Chapter IV, my results showed that C-CaLITR 2.0 induces a significantly lower phagocytic activity compared to the splice variant, C-CaLITR 2.1. The latter induced a delayed phagocytic phenotype that increased significantly when using smaller bead targets. These results suggest that alternative splicing within the CYT region can fine-tune the functional output of immunoregulatory receptors belonging to the Immunoglobulin supper family (IgSF), which in this case involved the removal (or insertion) of an ITIM upstream of an ITSM. Moreover, these results suggest that CaLITR 2.1-mediated phagocytic activity can be uniquely mediated by an ITSM. However, unlike an ITAM, the ITSM contains only a single tyrosine residue unlikely capable of binding activating kinases such as Syk, which require a tandem of two tyrosine to bind its two SH2 domains for activation. This opens the question of what signalling molecules and pathways are involved in C-CaLITR 2.1-mediated phagocytosis. Signalling motifs such as the single tyrosine HemITAMs were shown to recruit Syk through dimerization with another identical HemITAM-containing receptor. This way, one SH2 domain of Syk can bind to a phosphorylated HemITAM on one receptor, and the other SH2 domain of Syk will bind to the HemITAM found in the proximal, identical receptor. This mechanism was shown for the human C-type lectin receptor, CLEC-2 (Hughes et al., 2010). In this chapter, I examined the potential signalling components that may have an effect on the phagocytic activity of C-CaLITR 2.1, including the roles of F-actin polymerization, PI3K, Src-Family Kinases (SFKs) and Syk.

First, I propose that C-CaLITR 2.1 mediates phagocytosis in a similar Syk-dependent fashion as HemITAM-containing receptors. I predict that inhibiting Syk using the

pharmacological inhibitor, ER 27319, will significantly diminish the phagocytic activity of C-CaLITR 2.1, suggesting a prominent role for Syk in this phenotype. I also hypothesize that C-CaLITR 2.1-mediated phagocytic capacity is dependent on other signalling molecules, such as PI3K and SFKs. The pharmacological inhibitor, wortmannin, is a non-specific PI3K inhibitor that was shown to inhibit phagocytosis in AD293 cells expressing the ITAM-bearing receptor, IpLITR 2.6b (Lillico et al., 2015). The engulfment of large particles (>3 μm) is highly reliant on PI3Ks, while the uptake of smaller particles (<3 μm) was shown to be unaffected by the selective deactivation of regulatory subunits of PI3K class I molecules (Vieira et al., 2001). Therefore, I predict a higher level of inhibition of C-CaLITR 2.1-mediated phagocytosis when stimulated with 4.5 μm YG versus 2.0 μm YG beads.

Src Family Kinases (SFKs) are a group of tyrosine kinases found in the cytoplasm that feature several distinct regions: an N-terminal domain unique to each member, a Src homology 3 (SH3) domain capable of binding to polyproline-rich sequences, a Src homology 2 (SH2) domain that attaches to phosphotyrosine residues, and a domain responsible for tyrosine kinase activity (Abram, 2008). They regulate signalling by phosphorylating tyrosine residues found within receptor CYT regions (i.e., ITAMs, ITIMs or ITSMs), which can then recruit other kinases or phosphatases, such as Syk or SHP-2, respectively. SFKs, such as Fyn and Lck, are indispensable in PD-1 phosphorylation and the subsequent recruitment of SHP-2 (Patsoukis et al., 2020). PD-1 plays a major role in T-cell regulation, specifically in facilitating programmed cell death. Therefore, I predict that by treating cells expressing C-CaLITR 2.1 with the SFK-specific inhibitor PP2, phagocytosis will significantly diminish. Pharmacological profiling of signalling pathways mediating phagocytosis has been conducted in our lab using a variety of small molecule inhibitors (Lillico et al., 2015). Specifically, the inhibition of signalling molecules such

as SFK, Syk, PI3K, Akt, Cdc42, RAC1/2/3, MEK1/2, and PDK1, all led to a reduced phagocytic activity when the IpLITR 2.6b receptors were activated in RBL-2H3 cells. In contrast, only the inhibition of SFK, Syk, and F-actin polymerization had an effect on reducing phagocytosis via IpLITR 1.1b. This suggests that the signalling mechanisms employed by IpLITR 1.1b are distinct from those of IpLITR 2.6b and involve a more limited array of signalling components to trigger phagocytosis. Similarly, C-CaLITR 2.1 is distinct from 2.6b and 1.1b, as well as the ITAM-containing C-CaLITR 6.1, due to its unique configuration of tyrosine residue (i.e., ITSM) in the cytoplasmic region. This unique arrangement may result in the activation of a different set of signalling molecules, contributing to its distinct phagocytic characteristics.

Overall, the aim of this chapter is to highlight the potential signalling pathways involved in the phagocytic phenotype observed for C-CaLITR 2.1. A pharmacological assessment was performed on C-CaLITR 2.1 to probe into the possible participation of signalling molecules and elements (Syk, SFKs, PI3K, and F-actin polymerization) typically linked with phagocytosis. My findings suggest C-CaLITR 2.1 utilizes an actin, PI3K and SFK-dependent pathway, but to a lesser degree, a Syk-dependent pathway, to phagocytose bead targets. These observations differ when C-CaLITR 2.1 is engaged with smaller versus larger beads. The data in this chapter offers a broad view of the potential underlying mechanisms exerted by an ITSM-containing receptor during the stimulation of phagocytosis.

5.2 Results

To examine the potential signalling molecules involved in CaLITR-mediated phagocytosis, pharmacological inhibitors of Syk, SFK, PI3K and F-actin polymerization were tested for their effects on CaLITR-mediated phagocytosis. The selection of the aforementioned molecules was informed based on insights from pharmacological assays carried out in our lab

concerning phagocytosis mediated by IpLITR 2.6b and IpLITR 1.1b (Lillico et a., 2015). The inhibitors used in this study are detailed in Table 3.2, which provides the names of the drugs, their specific targets within the cell, and the dosages applied. The raw phagocytosis data for these assays are shown in the Appendix, Fig 2. The percentage of bead-cell association of samples treated with the inhibitors did not dramatically decrease; making the following observations comparable to the assays in Chapter IV. The percent inhibition was calculated as 1 minus the % phagocytosis of the inhibitor-treated group divided by the % phagocytosis of the vehicle control group containing 0.5% DMSO. Cells expressing either C-CaLITR 2.0, C-CaLITR 2.1 or C-CaLITR 6.1 were pre-treated with each pharmacological inhibitor for 1 hour at 37°C. Since the phagocytic activity of C-CaLITR 2.1 and C-CaLITR 2.0 was significantly different using larger beads (4.5 μm) than that using smaller beads (2.0 μm), cells in these pharmacological inhibitor assays were stimulated with either 4.5 μm beads or 2.0 μm beads. Overall, these experiments were done to help discern the potential roles of Syk, SFKs and PI3K in the size-dependent phagocytic phenotype displayed by C-CaLITR 2.1-expressing cells.

All construct-expressing cells (i.e., C-CaLITR 2.0, C-CaLITR 2.1, and C-CaLITR 6.1) showed significant inhibition of phagocytosis at 30 minutes and 60 minutes when treated with cytochalasin D (CytoD), compared to the vehicle control (Fig. 5.1, 5.2, and 5.3). CytoD inhibits the F-actin polymerization machinery that is required for plasma membrane remodelling during target capture and engulfment. For example, C-CaLITR 6.1 treated with CtyoD and exposed to 4.5 μm bead targets have diminished phagocytic activity by 87.7% ($p \leq 0.0005$) and 84.6% ($p \leq 0.00005$), at 30 and 60 minutes, respectively (Fig. 5.3A and 5.2B). In comparison, when using the 2.0 μm beads, percent inhibition of C-CaLITR 6.1 in response to CytoD pre-treatment is 86.5% ($p \leq 0.005$) and 87.4% ($p \leq 0.005$), at 30 and 60 minutes, respectively (Fig. 5.3A and

5.3B). When pre-treated by other inhibitors, such as wortmannin (PI3K), PP2 (SFKs), and ER 27319 (Syk), C-CaLITR 6.1-mediated phagocytosis differed significantly from the vehicle control at 30 ($p \leq 0.05$) and 60 ($p \leq 0.005$) minutes, but only when activated by small bead targets. Values of % inhibition for wortmannin (PI3K), PP2 (SFKs), and ER 27319 (Syk) ranged from 26.5% to 33.8% at 30 minutes (Fig. 5.3A), and after 60 minutes, it ranged from 27.1% to 37.0% inhibition of phagocytosis (Fig. 5.3B).

For cells expressing C-CaLITR 2.1, the inhibition profile showed a similar trend for experimental groups synchronized with either 4.5 μm or 2.0 μm bead targets, across both tested time points (30 and 60 minutes) (Fig. 5.2A and 5.2B). With 4.5 μm beads at 30 minutes, the phagocytic activity of C-CaLITR 2.1 was decreased by 80.3% ($p \leq 0.005$), 68.4% ($p \leq 0.005$), 55.3% ($p \leq 0.05$), and 22.6% ($p \leq 0.05$), when cells were pre-treated with CytoD, wortmannin, PP2, and ER 27319, respectively (Fig. 5.2A). At 60 minutes, % inhibition values were 84.3% ($p \leq 0.005$), 71.2% ($p \leq 0.0005$), 58.7% ($p \leq 0.00001$), and 16.6% ($p \leq 0.05$) when cells were pre-treated with CytoD, wortmannin, PP2, and ER 27319, respectively (Fig. 5.2B). In comparison, phagocytosis of 2.0 μm bead targets by C-CaLITR 2.1 expressing cells shows 77.9% ($p \leq 0.005$), and 54.4% ($p \leq 0.005$) inhibition at 30 minutes (Fig. 5.2A), and 75.4% ($p \leq 0.005$), and 56.0% ($p \leq 0.005$) inhibition at 60 minutes (Fig. 5.2B) when cells were pre-treated with CytoD and PP2. However, wortmannin pre-treatment for C-CaLITR 2.1 with 2.0 μm beads showed a significant difference from the DMSO vehicle control at both 30 and 60 minutes, with % inhibition values of 51.3% (ns) and 41.4% (ns), respectively (Fig. 5.2A and 5.2B). Similarly, pre-treatment with the Syk inhibitor, ER 27319, C-CaLITR 2.1 with 2.0 μm beads showed 6.0% (ns) inhibition at 30 minutes (Fig. 5.2A) and higher overall phagocytic response with a -7.1% ($p \leq 0.005$) inhibition at 60 minutes (Fig. 5.2B).

Since the phagocytic activity of C-CaLITR 2.0 was significantly less than both C-CaLITR 2.1 and C-CaLITR 6.1, the inhibition profile of this construct did not have significant differences from the vehicle control when engaged with either small or large beads at 30 or 60 minutes. Values of % inhibition ranged from -11.8% to 8.0% at 30 minutes (Fig. 5.1A). However, at 60 minutes, C-CaLITR 2.0 cells treated with PP2 and 2.0 μm showed a statistically higher ($p \leq 0.05$) % inhibition (Fig. 5.1B). Finally, C-CaLITR 2.0 treated with CtyoD and exposed to 4.5 μm bead targets have diminished phagocytic activity by 43.0% ($p \leq 0.05$) and 57.4% ($p \leq 0.05$), at 30 and 60 minutes, respectively (Fig. 5.1A and 5.1B). In comparison to 2.0 μm, the percent inhibition of C-CaLITR 2.0 in response to CytoD pre-treatment is 70.0% ($p \leq 0.05$) and 75.1% ($p \leq 0.005$), at 30 and 60 minutes, respectively (Fig. 5.1A and 5.1B).

5.3 Discussion

The primary aim of this chapter was to highlight the potential signalling molecules that drive phagocytosis upon C-CaLITR 2.1 engagement. Specifically, I examined the effect of molecules and elements (i.e., Syk, PI3K, SFKs, and F-actin polymerization) that are known to play a role in phagocytosis (Uribe-Querol & Rosales, 2020). This was done by pre-treating C-CaLITR-expressing cells with pharmacological inhibitors specific to those molecules. Subsequently, phagocytosis was carried out as described in section 3.5.1. Finally, % inhibition was calculated by comparing inhibitor-treated cells with samples that lacked an inhibitor. Raw phagocytosis percentages of these assays are shown in the Appendix Figure 2. Overall, my results show that C-CaLITR 2.1 signals through SFK and PI3K-dependent pathways in facilitating phagocytosis in AD293 cells. It also seems to require Syk but to a lesser extent than SFK or PI3K. Furthermore, the inhibitory effects on phagocytosis varied when the receptor interacts with smaller versus larger bead targets, and this was particularly evident for PI3K.

In Chapter IV, examining the phagocytic activity induced by C-CaLITR 2.0 and its splice variant, C-CaLITR 2.1, led to the observation that C-CaLITR 2.1 generates a size-dependent and delayed phagocytic response. These results provided evidence for the functional consequences of alternative splicing of CYT-specific regions in teleost immunoregulatory receptors. However, the signalling components driving this process are unknown. This phenotype is unlike that mediated by an ITAM-containing receptor, such as C-CaLITR 6.1, as its maximal phagocytosis occurs at a later time point (30 minutes) of beads-cell interactions. In addition, the phenotype seems to be size-dependent as C-CaLITR 2.1 shows higher phagocytic activity upon engagement of small-size beads versus larger ones, an observation not seen by the ITAM-bearing C-CaLITR 6.1. Collectively, these novel phenotypic differences between classical phagocytic receptors (i.e.., C-CaLITR 6.1) vs. those containing a switch tyrosine motif (i.e., ITSM) offers the question regarding what signalling components are being utilized by C-CaLITR 2.1 during phagocytosis. Thus, the next logical step in this research was to profile common signalling elements, specifically Syk, PI3K, SFKs, and F-actin polymerization, that were shown to modulate phagocytosis in multiple different contexts (Lillico et al., 2015). This general approach was adapted due to the complexity of immunoregulatory receptor signalling networks. It is difficult to predict which signalling molecules play a more prominent role in the phagocytic activity of C-CaLITR 2.1, especially since it contains a CYT motif (i.e., ITSM) that is relatively less studied than the classical ITAMs and ITIMs (Liu et al., 2015). Therefore, the use of pharmacological blockers for signal transduction molecules connected with diverse receptor signalling cascades enabled a broad-based approach to identify the signalling agents that C-CaLITR 2.1 might use to induce phagocytosis. Pharmacological drugs were previously applied to delineate the signalling pathways activated by IpLITR 1.1b. When this receptor was expressed in myeloid cells, such as

RBL-2H3, it triggered a distinct phagocytic mechanism that did not depend on ITAMs and was only disrupted by inhibitors targeting Syk, SFK, and the polymerization of F-actin (Lillico et al., 2015). In contrast, the phagocytic activity mediated by the ITAM-bearing receptor IpLITR 2.6b was susceptible to drugs that inhibit Syk, SFK, F-actin polymerization, PI3K, PDK1, Cdc42, Rac, and PKCs. While this prior application of pharmacological inhibitors to study phagocytosis was premised on the expression of IpLITRs in RBL-2H3 cells, similar experiments were conducted with IpLITR and DrLITR in AD293 cells (Niemand & Stafford, 2023). Findings from these experiments confirm that pharmacological inhibition of common signalling molecules indeed translates across cell lines. Specifically, the suppression of SFKs, Syk, PI3K, and F-actin polymerization all played a significant role in reducing IpLITR 2.6b-induced phagocytosis in AD293 cells in a fashion similar to that observed in RBL-2H3 cells (Niemand & Stafford, 2023; Lillico et al., 2015). Finally, the concentrations of inhibitors used in my experiments were initially determined based on the inhibitors assays conducted in the AD293 system, as well as preliminary experiments on the phagocytic receptor C-CaLITR 6.1.

Cytochalasins, are a group of compounds produced by fungi that impact a broad array of movement-related functions in eukaryotic cells. They are known to obstruct the polymerization of actin, likely because they bind strongly to the expanding ends of actin nuclei and filaments (F-actin), thus blocking the attachment of monomeric units (G-actin) at those points (Casella et al., 1981). Using a quantitative high-resolution confocal microscopy technique it was shown that treatment of AD293 cells (expressing the N-terminal myc-tagged serotonin$_{1A}$ receptor) with 5 μM cytochalasin D resulted in the loss of F-actin filaments and formation of F-actin aggregates (Shrivastava et al., 2020). F-actin polymerization is necessary to facilitate membrane remodelling events that lead to particle internalization (Jaumouillé & Waterman, 2020).

Therefore, I predicted that all of C-CaLITR 2.0, C-CaLITR 2.1 and C-CaLITR 6.1 would show significant inhibition of phagocytosis when pre-treated with cytochalasin D. Indeed, phagocytosis decreased dramatically at 30 and 60 minutes, for all constructs-expressing cells, when stimulated with either small (2 μm) or large (4.5 μm) beads (Fig 5.1, 5.2 and 5.3). This suggests that the phagocytic phenotypes observed by both C-CaLITR 2.1 and C-CaLITR 6.1 are actin-dependent, which is consistent with our current understanding that actin polymerization is essential for target engulfment (Jaumouillé & Waterman, 2020). The inhibitory effects of cytochalasin D are well studied and are shown across different cell types, receptors, and antigens (Ribes et al., 2010; Kapetanovic et al., 2007). For example, in a study where monocyte-derived macrophages (MDMs) were fed osmium-stained *E. coli* DH5-*α* cells (1.0-2.0 μm), the number of phagocytosed bacteria after 60 minutes decreased by 70% when macrophages were pre-treated with cytochalasin D (Schulz et al., 2019). In our lab, inhibition of phagocytosis by AD293 cells expressing IpLITR 2.6b or DrLITR 1.2 ranged from 84-90% (Niemand & Stafford, 2023). Overall, this set of experiments indicates that the delayed, and size-dependent phagocytic phenotype of C-CaLITR 2.1 is actin-dependent.

Wortmannin, derived from the fungi *Talaromyces wortmannii,* is a broad-spectrum, irreversible inhibitor of phosphoinositide 3-kinases (PI3Ks) (Vanhaesebroeck et al., 2001). Wortmannin has equivalent effectiveness against classes I, II, and III of PI3K, yet at elevated concentrations, it may also affect other PI3K-related enzymes, protein kinase C (PKC), certain phosphatidylinositol 4-kinases, myosin light chain kinase (MLCK), and mitogen-activated protein kinase (MAPK) (Ferby et al., 1994; Vieira et al., 2001). In mammals, Fc receptor (FcR)-mediated phagocytosis unfolds in distinct stages that rely on the coordinated action of various critical effector molecules (Rougerie et al., 2013). The initial phase involves a PI3K-independent

prompt of actin polymerization, which propels the formation of phagocytic cups to surround target particles, followed by a PI3K-dependent constriction mechanism that seals the phagosomes around the targets. As such, it was hypothesized that C-CaLITR-mediated phagocytosis, measured by the percentage of cells exhibiting the complete internalization of at least one particle, is dependent on PI3K activity. Indeed, the phagocytic activity of C-CaLITR 2.1 was significantly diminished at 30 and 60 minutes, when pre-treated with wortmannin (Fig. 5.2A and 5.2B). In addition, PI3K signalling is implicated in size-dependent phagocytosis (Araki et al., 1996). For example, when IgG-opsonized beads ranging from 1 to 6 μm were used as targets for murine macrophages (i.e., RAW LacR/FMLP.2 cells), the effectiveness of PI3K inhibition on phagocytosis was dependent on the size of the beads (Cox et al., 1999). Most notably, wortmannin (10 nM) inhibited macrophage phagocytic activity by ~50% when incubated with 2 μm beads, compared to 90% when incubated with 4.5 μm beads. Previously, in Chapter IV, I showed that C-CaLITR 2.1 induced a significantly higher phagocytic response against small beads than larger beads; therefore, in this chapter, I was interested to see if this discrepancy is related to PI3K inhibition. Indeed, the inhibition of phagocytosis, based on PI3K blockade using wortmannin, is dependent on the particle size. For example, C-CaLITR 2.1-mediated phagocytosis diminished by 41.4% (ns) when cells were engaged with 2 μm beads. Meanwhile, wortmannin inhibited phagocytosis by 71.2% ($p \leq 0.0005$) when cells were stimulated with 4.5 μm beads (Fig 5.2A). This difference in the magnitude of PI3K inhibition (~30%) of phagocytosis of small vs large beads was only observed for C-CaLITR 2.1. The ITAM-bearing phagocytic receptor, C-CaLITR 6.1, showed relatively similar inhibition values (ranging from 27-38%) when stimulated with either size of bead targets (Fig. 5.3A). The reason for this discrepancy is currently unknown, but it could possibly be related to the CYT signalling

motif (i.e., ITSM) that kickstarts the signalling cascade. For example, ITSM-mediated signalling involves different key molecules (i.e., Fyn, SAP, EAT-2, SHP-2) than that of ITAM-mediated pathways (i.e., PI3K, PDK1, Cdc42, Rac, and PKCs). PI3K levels in the cell might also affect those observations and require further examination upon stimulation with different beads. Overall, this set of experiments provides evidence of the important role that PI3K plays in C-CaLITR 2.1 delayed and size-dependent phagocytosis.

SFKs are cytoplasmic tyrosine kinases involved in the proximal initiation of signalling cascades associated with a wide range of effector responses such as cell growth and differentiation, cytokine secretion and phagocytosis (Parsons & Parsons, 2004; Majeed et al., 2001). They work by phosphorylating the tyrosine motifs (i.e., ITAMs, ITIMs, or ITSMs) found in the CYT region of membrane-bound receptors, which can subsequently recruit other kinases (e.g., Syk, ZAP-70) or phosphatases (e.g., SHP-1, SHP-2) to either activate or dampen cellular functions. C-CaLITR 2.1 contains an ITSM, and phosphorylated ITSMs were shown to recruit signalling molecules such as SHP-2 and SLAM family-associated proteins (SAP) such as Ewing's sarcoma-activated transcript-2 (EAT-2) and EAT-2-related transducer (ERT) (Dragovich & Mor, 2018; Farhangnia et al., 2023). However, the recruitment of SH2 domain-containing adaptor molecules is dependent on the phosphorylation of the receptor-bound tyrosine motif by an SFK, such as Fyn or Lck (Bottino et al., 2001; Patsoukis et al., 2020). Therefore, I expected to see a significant reduction in the phagocytic activity mediated by C-CaLITR 2.1 when pre-treated with the SFK inhibitor, PP2. Indeed, inhibition levels ranged from 52-58% and were consistent at 30 and 60 minutes of incubation with either small or large beads. (Fig. 5.2A and 5.2B). Overall, this suggests that: i) C-CaLITR 2.1-mediated phagocytosis is dependent on the activity of membrane-proximal SFKs, and ii) AD293 cells contain the necessary repertoire of

SFKs that can potentially phosphorylate an ITSM motif. In a study that was done to examine the signalling components involved in the programmed cell death-1 (PD-1) receptor function, Fyn was shown to be necessary to phosphorylate the tyrosine (i.e., Y^{248}) found within the ITSM (Patsoukis et al., 2020). Consequently, the phosphorylated tyrosine (pY^{248}) was able to recruit SHP-2 upon PD-1 engagement, ultimately resulting in inhibiting T-cell IL-2 production. When the ITSM was mutated or Fyn was inactivated, PD-1 did not associate with SHP-2 nor inhibit T-cell function (Patsoukis et al., 2020). Similarly, SFKs seem to be important for C-CaLITR 2.1-mediated phagocytosis, and the ITSM within the CYT region of 2.1 is potentially a target of SFK-phosphorylation that then functions as a site to recruit SH2 domain-containing adaptors to propagate further downstream signaling. The establishment of the precise role of ITSM would require further studies, specifically functional assays of an ITSM mutant and protein-protein interaction studies. Overall, the inhibition assays of SFK add more to our understanding of the signalling network underlying C-CaLITR 2.1 unique phagocytic capacity.

Findings from the experiments above point to actin, PI3K, and SFK-dependent mechanism for C-CaLITR 2.1-mediated phagocytosis. However, the inhibitors against these signalling components take effect at the distal stage of the phagocytic response (PI3K engagement and F-actin polymerization) or target a range of kinases (SFKs) at the proximal stage without pin-pointing the specific molecule responsible for the delayed and size-dependent response. Therefore, inhibition assays with the Syk inhibitor, ER 27319, were also performed. Syk is a well-studied activator of Fc-receptor-mediated phagocytosis, which it does by binding to a tandem of two phosphorylated tyrosines found within an ITAM embedded in the CYT of membrane-bound receptors (Kiefer et al., 1998). This interaction requires the binding of both SH2 domains on Syk for it to be activated, and then subsequently to activate other downstream

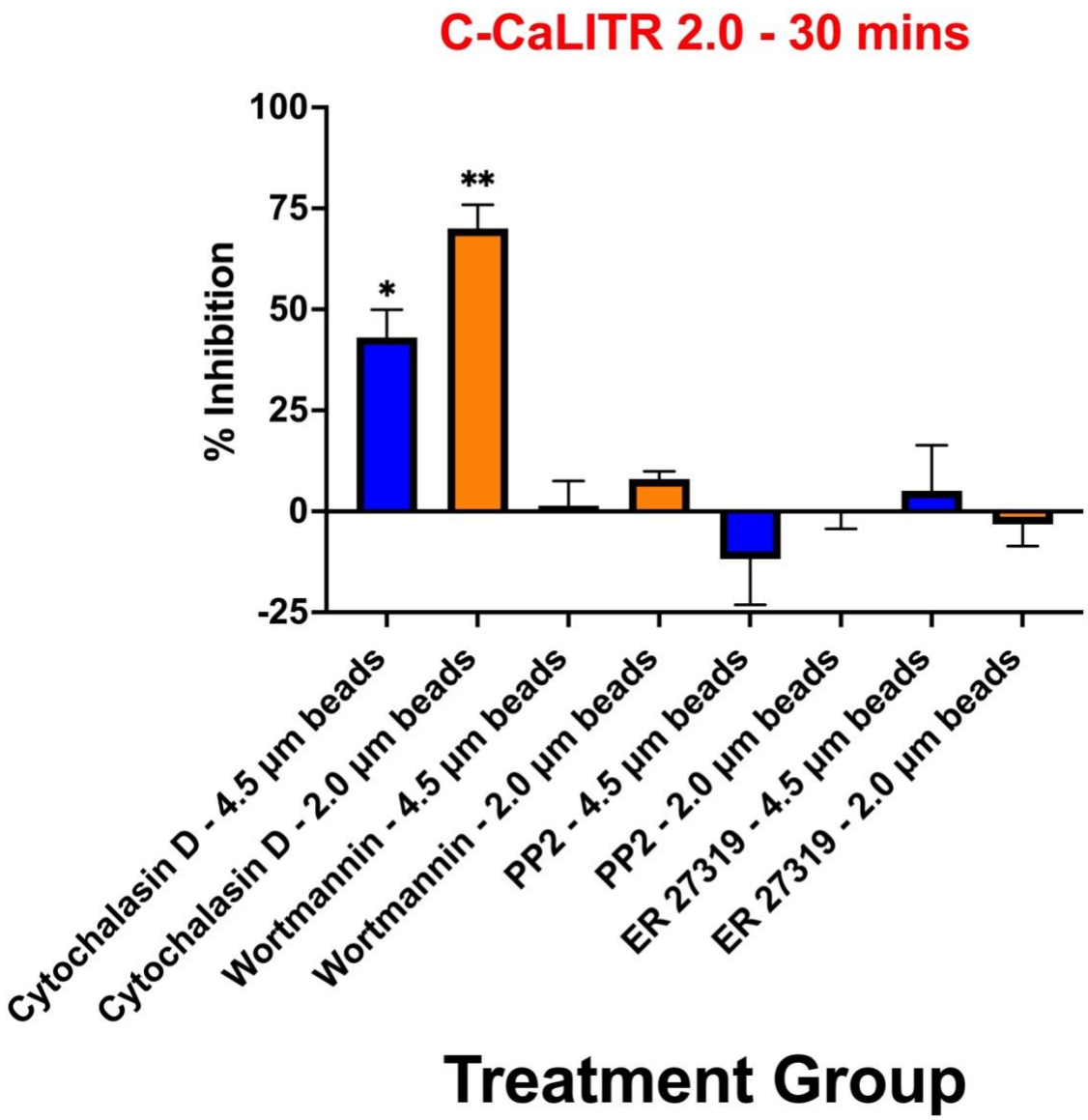
molecules. In contrast to ITAMs, ITSMs contain only a single tyrosine residue (i.e. Y^{252} in C-CaLITR 2.1). This makes it unlikely that an ITSM recruits Syk or other kinases or phosphatases that contain two SH2 domains. Nevertheless, signalling motifs such as HemITAMs, which have a single tyrosine, can still recruit Syk by pairing with another identical receptor that also contains a HemITAM (Rogers et al., 2005). In this arrangement, the N-terminal SH2 domain of Syk attaches to a phosphorylated HemITAM on one receptor, while the C-terminal SH2 domain attaches to the HemITAM on an adjacent, identical receptor. This mechanism was observed for C-type lectin receptors such as CLEC-2 and Dectin-1 (Rogers et al., 2005; Underhill et al., 2005).

Receptor-dimerization, or aggregation at the site of particle engagement, is a common phenomenon that leads to signal amplification (Fallahi-Sichani & Linderman, 2009). Perhaps, multiple C-CaLITR 2.1 proteins are crosslinked on the cell membrane or move to the lipid raft upon binding to anti-HA-opsonized beads. This would bring the receptors into close proximity where the two SH2 domains of Syk, for example, can dock onto the phosphorylated ITSMs of two identical C-CaLITR 2.1s. The likelihood of this hypothetical mechanism is not far-fetched for an ITSM-containing receptor that induces an activating functional output (i.e., phagocytosis) in a novel, ITAM-independent fashion. Therefore, I hypothesized that inhibiting Syk, using ER 27319, would result in a significant reduction of C-CaLITR 2.1-mediated phagocytosis. While my findings show a significant difference between the ER 27319-treated cells and the control when stimulated with 4.5 μm beads (Fig. 5.2A and 5.2B), the mean inhibition values from three different trials were only 22.7% and 16.6% at 30 and 60 minutes, respectively. For reference, inhibition of PI3K and SFKs resulted in 3x and 2x less phagocytosis, respectively (Fig. 5.2A and 5.2B). In addition, when ER 27319-treated cells were engaged with 2.0 μm beads, no significant

difference between the negative controls was observed. While these results suggest a minimal, yet possible, role of Syk in the induction of phagocytosis by C-CaLITR 2.1, that cannot be concluded without verifying Syk recruitment through staining and visualization techniques of the phagocytic cup triggered by C-CaLITR 2.1 signalling. In addition, protein-protein interaction studies would help determine if Syk or any other potential signalling molecule associates with C-CaLITR 2.1 after receptor engagement.

Overall, the pharmacological assays provided useful insights into the possible signalling pathways utilized by C-CaLITR 2.1 to induce its unique form of delayed and size-dependent phagocytosis. I showed that C-CaLITR 2.1 drives a phagocytic response that is actin-dependent, PI3K-dependent, SFK-dependent, and to a lesser degree, may involve Syk. I also showed that inhibition of PI3K is more detrimental to C-CaLITR 2.1-mediated engulfment of larger beads, and less so of smaller beads. While it is difficult to propose a full-fledged mechanism on how C-CaLITR 2.1 modulates phagocytosis, pharmacological studies provide a good foundation to examine which specific signalling molecules are driving this unique phenotype.

A.


C-CaLITR 2.0 - 30 mins
100
75
50
25
0
-25
% Inhibition
*
**
Cytochalasin D - 4.5 µm beads
Cytochalasin D - 2.0 µm beads
Wortmannin - 4.5 µm beads
Wortmannin - 2.0 µm beads
PP2 - 4.5 µm beads
PP2 - 2.0 µm beads
ER 27319 - 4.5 µm beads
ER 27319 - 2.0 µm beads
Treatment Group

B.

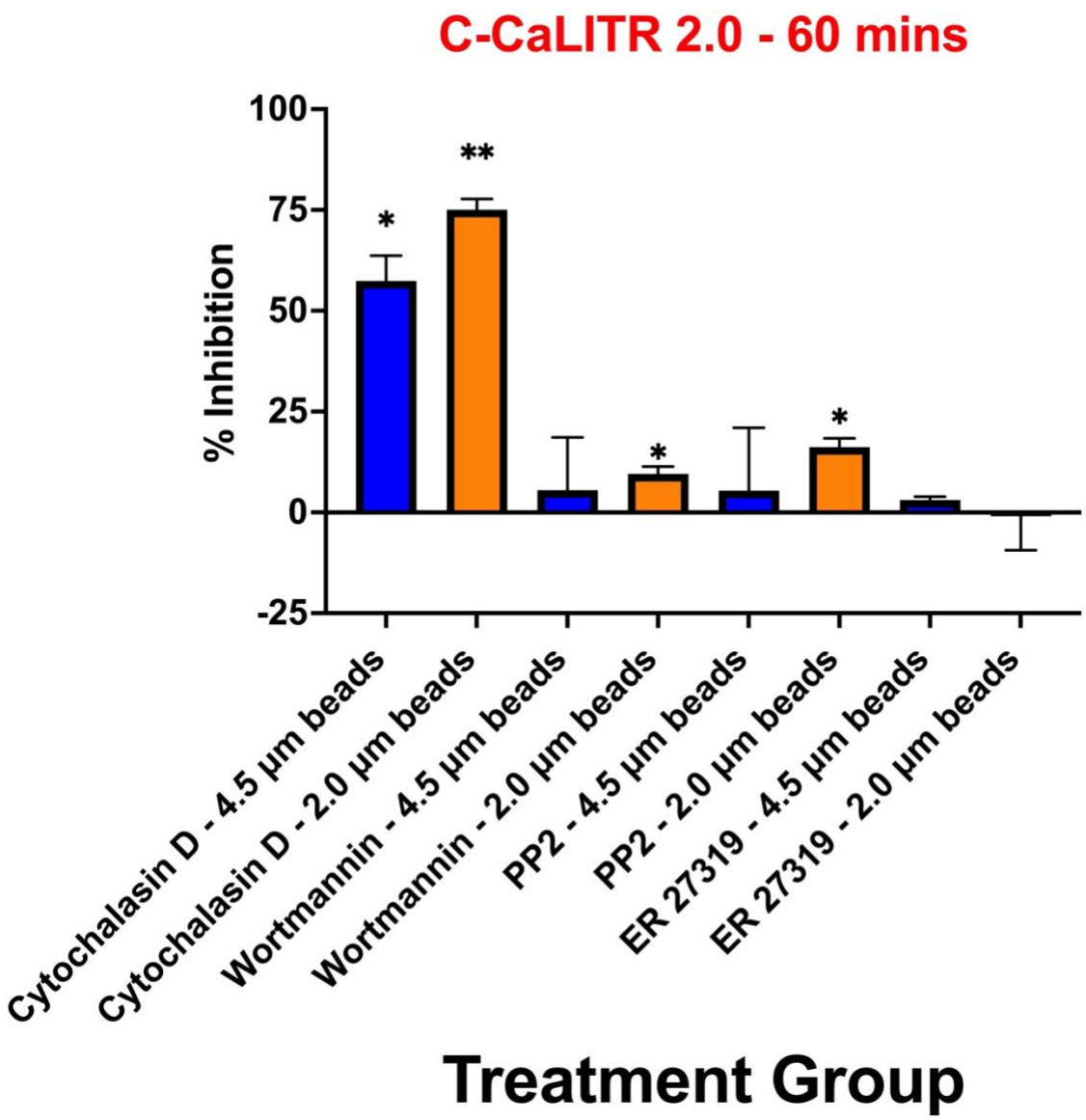
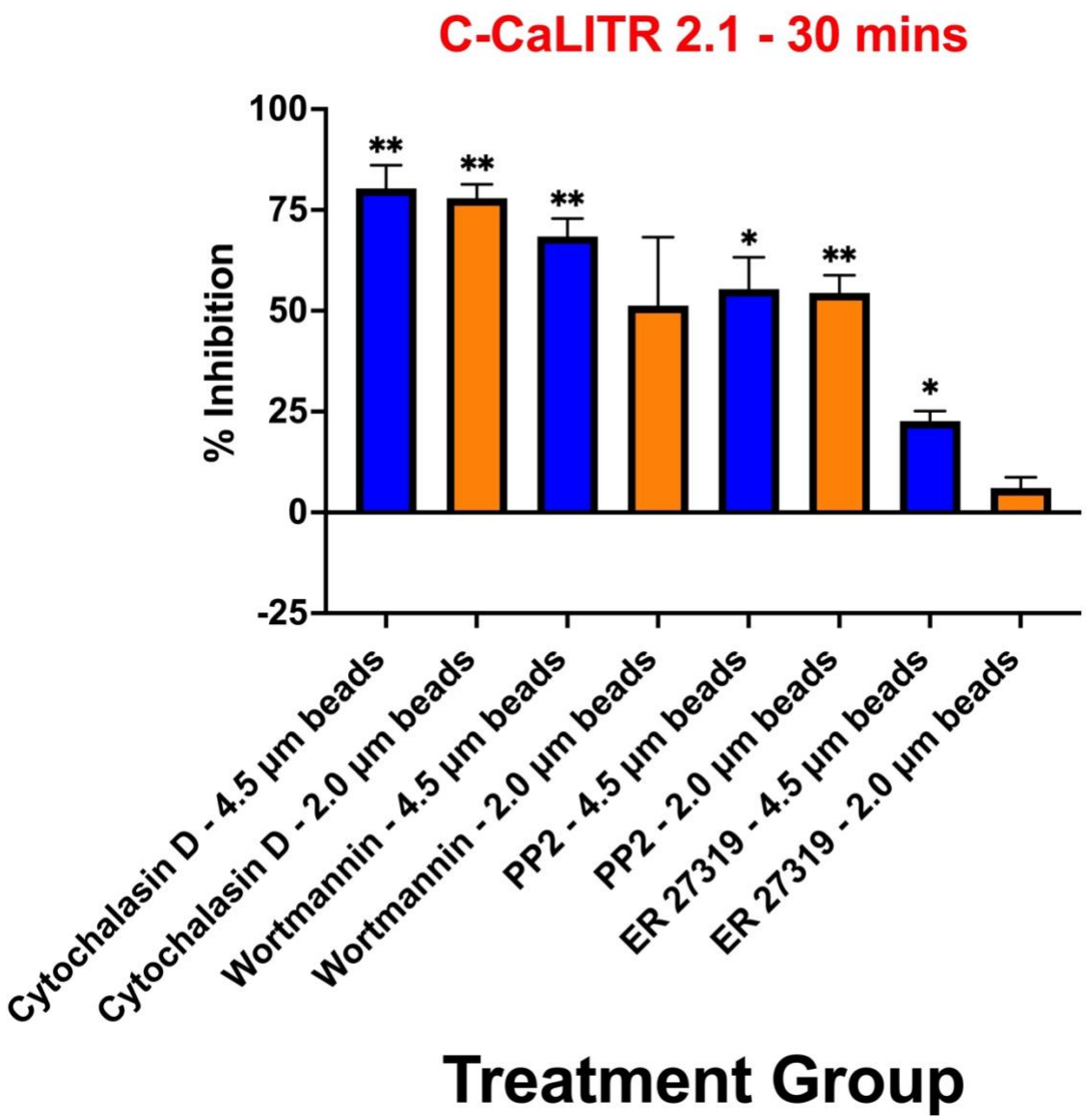

C-CaLITR 2.0 - 60 mins
100
75
50
25
0
-25
% Inhibition
*
**
*
*
Cytochalasin D - 4.5 µm beads
Cytochalasin D - 2.0 µm beads
Wortmannin - 4.5 µm beads
Wortmannin - 2.0 µm beads
PP2 - 4.5 µm beads
PP2 - 2.0 µm beads
ER 27319 - 4.5 µm beads
ER 27319 - 2.0 µm beads
Treatment Group

Figure 5.1. Pharmacological inhibition during C-CaLITR 2.0-mediated Phagocytosis. 3 x 10^5 AD293 cells stably expressing C-CaLITR 2.0 constructs were preincubated for 1 hour with pharmacological blockers or with 0.5% DMSO vehicle control. Without removing drug inoculated media, samples were incubated with either 4.5μm (blue) or 2.0μm (orange) YG beads (9 x 10^5) opsonized with 2 μg/mL mAb αHA for (**A**) 30 or (**B**) 60 minutes at 37°C. Samples were then counter-stained and subsequently analyzed using the ImageStream X Mark II, with samples classified as being either phagocytic or surface-bound for YG bead targets. Inhibition % was calculated as (1 - % phagocytosis experimental group / % phagocytosis vehicle control group). Each bar represents the mean ± SEM of % inhibition from 3 separate experiments. Sample data groups were analyzed using a t-test (Prism 10, GraphPad, La Jolla, CA, USA). Asterisks (*) represent statistical significance ($p \leq 0.05$) between the analyzed sample and the DMSO vehicle control.

A.


C-CaLITR 2.1 - 30 mins
% Inhibition
100
75
50
25
0
-25
**
**
**
*
**
*
Cytochalasin D - 4.5 µm beads
Cytochalasin D - 2.0 µm beads
Wortmannin - 4.5 µm beads
Wortmannin - 2.0 µm beads
PP2 - 4.5 µm beads
PP2 - 2.0 µm beads
ER 27319 - 4.5 µm beads
ER 27319 - 2.0 µm beads
Treatment Group

B.

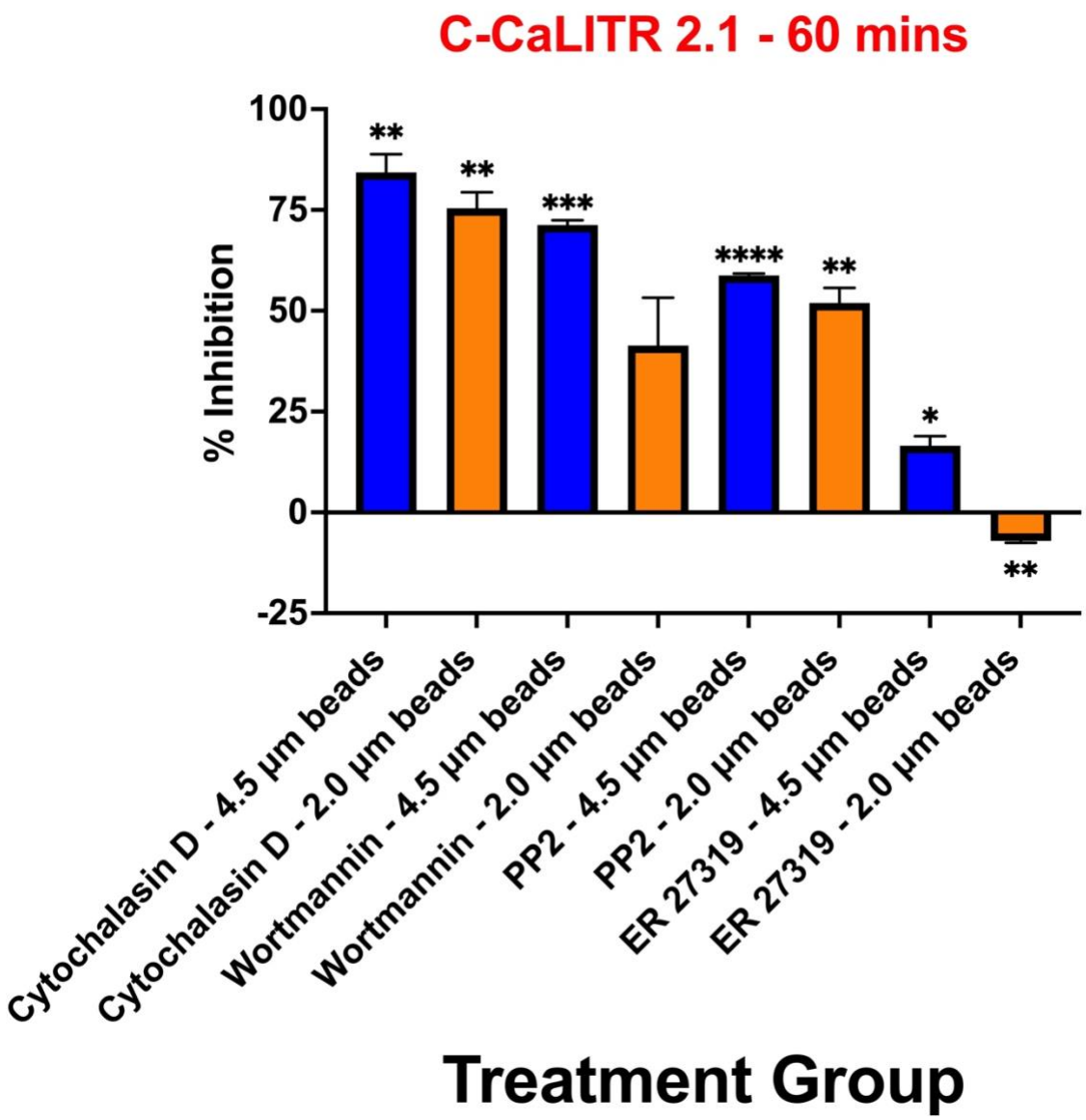
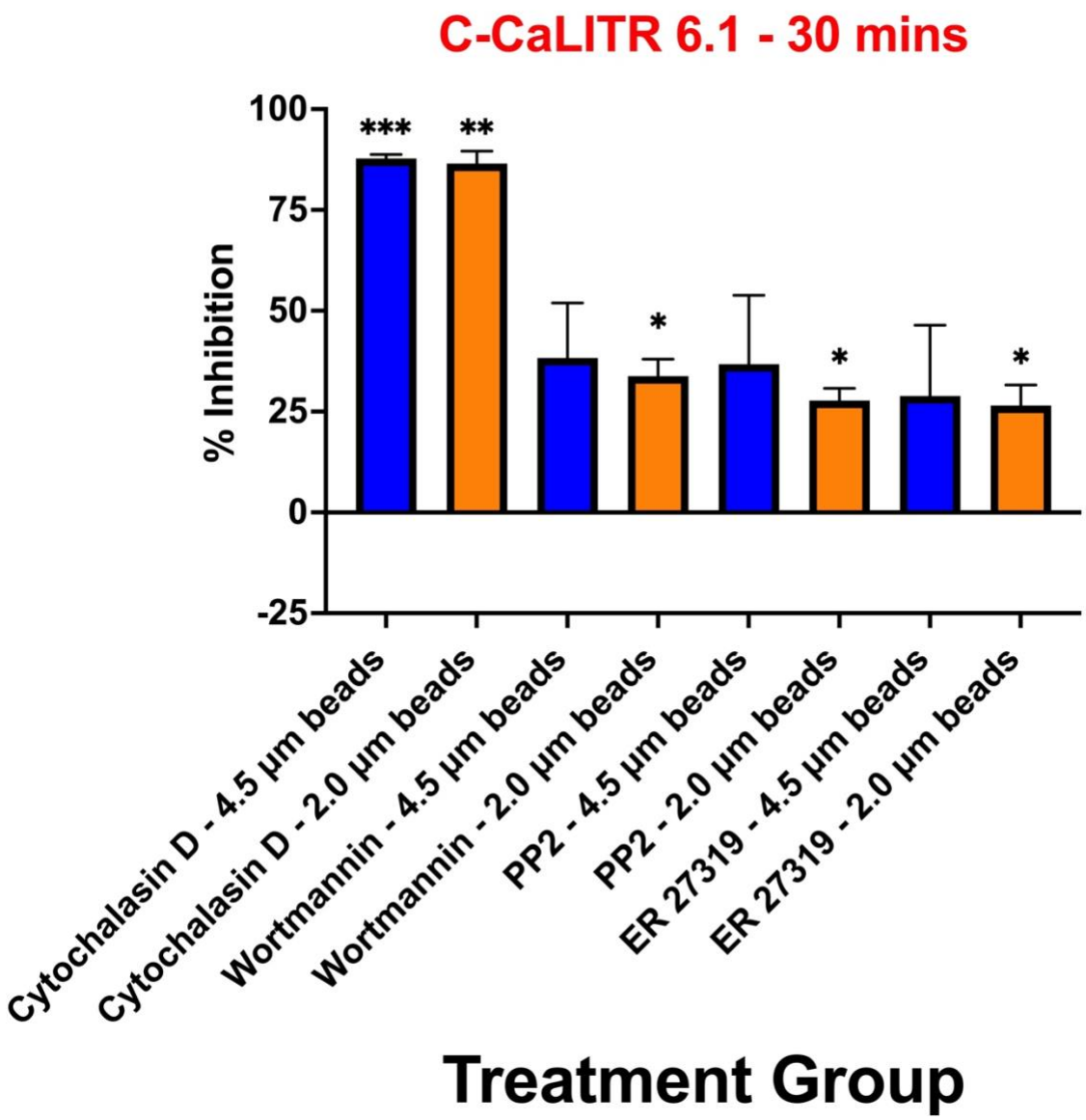

C-CaLITR 2.1 - 60 mins
% Inhibition
100
75
50
25
0
-25
**
**
***
****
**
*
**
Cytochalasin D - 4.5 µm beads
Cytochalasin D - 2.0 µm beads
Wortmannin - 4.5 µm beads
Wortmannin - 2.0 µm beads
PP2 - 4.5 µm beads
PP2 - 2.0 µm beads
ER 27319 - 4.5 µm beads
ER 27319 - 2.0 µm beads
Treatment Group

Figure 5.2. Pharmacological inhibition during C-CaLITR 2.1-mediated Phagocytosis. 3 x 10^5 AD293 cells stably expressing C-CaLITR 2.1 constructs were preincubated for 1 hour with pharmacological blockers or with 0.5% DMSO vehicle control. Without removing drug inoculated media, samples were incubated with either 4.5μm (blue) or 2.0μm (orange) YG beads (9 x 10^5) opsonized with 2 μg/mL mAb αHA for (**A**) 30 or (**B**) 60 minutes at 37°C. Samples were then counter-stained and subsequently analyzed using the ImageStream X Mark II, with samples classified as being either phagocytic or surface-bound for YG bead targets. Inhibition % was calculated as (1 - % phagocytosis experimental group / % phagocytosis vehicle control group). Each bar represents the mean ± SEM of % inhibition from 3 separate experiments. Sample data groups were analyzed using a t-test (Prism 10, GraphPad, La Jolla, CA, USA). Asterisks (*) represent statistical significance ($p \leq 0.05$) between the analyzed sample and the DMSO vehicle control.

A.


C-CaLITR 6.1 - 30 mins
% Inhibition
100
75
50
25
0
-25
***
**
*
*
*
Cytochalasin D - 4.5 µm beads
Cytochalasin D - 2.0 µm beads
Wortmannin - 4.5 µm beads
Wortmannin - 2.0 µm beads
PP2 - 4.5 µm beads
PP2 - 2.0 µm beads
ER 27319 - 4.5 µm beads
ER 27319 - 2.0 µm beads
Treatment Group

B.

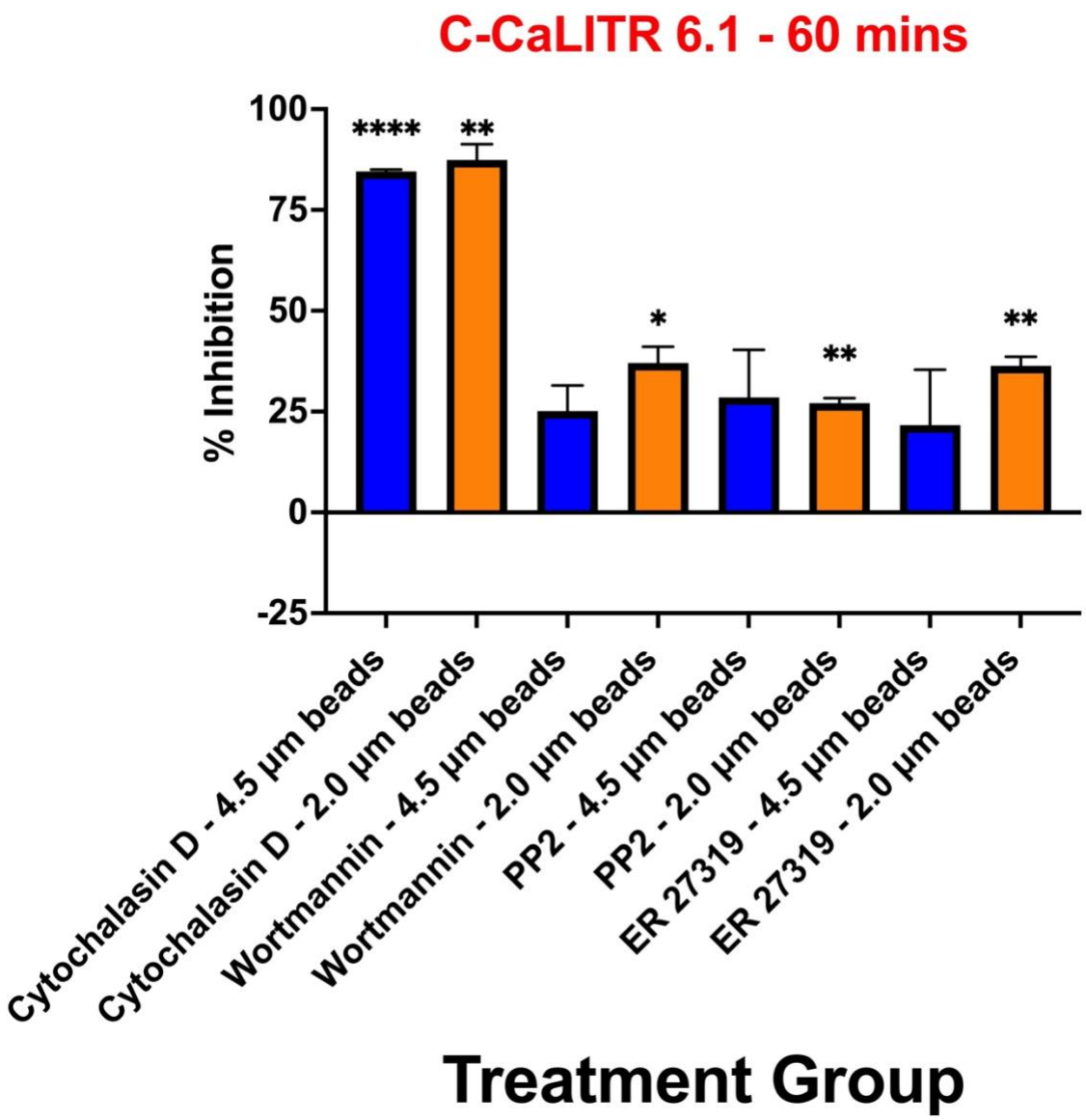

C-CaLITR 6.1 - 60 mins
% Inhibition
100
75
50
25
0
-25
****
**
*
**
**
Cytochalasin D - 4.5 µm beads
Cytochalasin D - 2.0 µm beads
Wortmannin - 4.5 µm beads
Wortmannin - 2.0 µm beads
PP2 - 4.5 µm beads
PP2 - 2.0 µm beads
ER 27319 - 4.5 µm beads
ER 27319 - 2.0 µm beads
Treatment Group

Figure 5.3. Pharmacological inhibition during C-CaLITR 6.1-mediated Phagocytosis. 3 x 10^5 AD293 cells stably expressing C-CaLITR 6.1 constructs were preincubated for 1 hour with pharmacological blockers or with 0.5% DMSO vehicle control. Without removing drug inoculated media, samples were incubated with either 4.5μm (blue) or 2.0μm (orange) YG beads (9 x 10^5) opsonized with 2 μg/mL mAb αHA for (**A**) 30 or (**B**) 60 minutes at 37°C. Samples were then counter-stained and subsequently analyzed using the ImageStream X Mark II, with samples classified as being either phagocytic or surface-bound for YG bead targets. Inhibition % was calculated as (1 - % phagocytosis experimental group / % phagocytosis vehicle control group). Each bar represents the mean ± SEM of % inhibition from 3 separate experiments. Sample data groups were analyzed using a t-test (Prism 10, GraphPad, La Jolla, CA, USA). Asterisks (*) represent statistical significance ($p \leq 0.05$) between the analyzed sample and the DMSO vehicle control.

CHAPTER VI

GENERAL DISCUSSION AND FUTURE DIRECTIONS

6.1 Summary of Findings

The recent identification of leukocyte immune-type receptors in goldfish (*Carassius Auratus*) has unveiled a new group of immunoglobulin proteins in fish for research into immune regulatory processes. CaLITRs show evolutionary, structural, and molecular similarities with members of the IgSF, and they also uniquely contain CYT region-splicing events (Wang et al., 2020). Specifically, CaLITR 2.1 is a splice variant of CaLITR 2.0 that lacks a sequence of 29 amino acids in its CYT, removing Y^{465}, potentially implicated in cell signalling. After original analysis of the receptor sequence, I hypothesized that CaLITR 2.0 contains an ITAM-like motif ([E]xxY^{465}xx[I]$x_{(25)}$$Y^{494}$xx[L]), displaying a nonconventional spacing of 25 amino acids between the two tyrosine pockets. Based on this, I proposed that CaLITR 2.0, when expressed on the surface of AD293 cells and engaged by mAb-opsonized beads, may induce phagocytosis through an ITAM-like driven signalling pathway. In contrast, the splice variant, CaLITR 2.1, which contains an ITSM ([D]xxY^{463}xx[L], was predicted to exhibit reduced phagocytic activity relative to CaLITR 2.0 due to the elimination of Y^{465} within its hypothesized ITAM-like motif. Such a modification in the CYT region distinctly differentiates these nearly identical receptors, creating one with an ITAM-like sequence to a variant possessing an ITSM only. As such, any functional discrepancies were predicted to result from an alternative splicing event within the CYT region (Fig. 4.2A). In essence, my overall hypothesis in this thesis is that the splicing of a tyrosine-containing region within the CYT domains of goldfish LITRs will influence their signalling capabilities and, in turn, their role in modulating immune effector functions, particularly phagocytosis.

I used the AD293 heterologous expression system (i.e., transfection and stable expression of *litrs* on the cell surface of AD293 cells) to examine the functional differences between CaLITR 2.0 and its splice variant, CaLITR 2.1. The previous use of this system in our lab has been instrumental in examining the phagocytic characteristics of IpLITRs, as well as in the more detailed study of the corresponding immune response signalling pathways (Cortes et al., 2012; Lillico et al., 2015; Fei et al., 2020). Unfortunately, neither CaLITR 2.0 nor 2.1 were expressed on the cell surface, and attempts to optimize the transfection protocol by altering the transfection reagent amount, the plasmid DNA amount, the amount of selection antibiotic used, and the time to evaluate expression or cell sorting, were all futile. To test if this effect is specific to CaLITR 2.0 and 2.1, CaLITR 4.1 and CaLITR 6.1 were similarly transfected but neither of these CaLITRs were expressed on the cell surface (Fig 4.1D). Sequencing of native CaLITRs using pDisplay primers shows that each construct is correctly in-frame (Appendix; Fig. 1), which means that the expression issue is not due to cloning errors. AD293 cells transfected with native CaLITR constructs were able to survive the Geneticin (G418, 400 ug/mL) antibiotic selection process, which suggests that the transfected cells were able to integrate, transcribe and translate the plasmid vector, specifically the gene conferring resistance to geneticin. Therefore, it seems that the inability of AD293 cells to express any native CaLITR construct (2.0, 2.1, 4.1 and 6.1) may be an inherent property of the receptors. As such, I theoretically investigated a few potential motifs (e.g., KDEL, double arginine [RRxx], and double lysine [KKxx]) that may cause the retention of native CaLITRs within the cellular trafficking system of AD293 cells, ultimately preventing their cell surface expression. None provided an explanation as to why native CaLITRs are not being expressed. Therefore, instead of experimentally mutating potential retention motifs and transfecting mutant CaLITRs, I opted to use chimeric (C) CaLITR

constructs. These constructs were created by fusing the extracellular Ig-like domains and TM segment of IpLITR 2.6b with the CYT regions from each of CaLITR 2.0, 2.1 and 6.1 (Fig 4.1C, 4.1D, and 4.1E). Since the objective of this thesis was to resolve the functional consequences of CYT region targeted splicing events, and since we are using a synthetic system in which constructs are tagged with an HA-epitope and engaged with anti-HA mAb antibodies, changing the extracellular and TM domains of the native CaLITRs is unlikely to affect the overall functional output initiated by the CYT of the newly created chimeras. Overall, this approach resulted in the successful expression of chimeric constructs containing the CYT regions of the goldfish receptors of interest for this study. For a positive control, C-CaLITR 6.1, which contains a bonafide ITAM and was previously shown to induce a potent phagocytic response (unpublished data), was also used in all phagocytosis assays.

In Chapter IV, I showed that C-CaLITR 2.0, which was initially thought to contain an ITAM-like sequence (Wang et al., 2020) and was hypothesized to induce a phagocytic response, induces a significantly lower phagocytic response than the positive control, C-CaLITR 6.1, and the splice variant, C-CaLITR 2.1 (Fig. 4.3D). As mentioned above, the typical spacing between tyrosines in an ITAM sequence ranges from 6 to 12 amino acids (Billadeau & Leibson, 2002). In the ITAM-like sequence of CaLITR 2.0 ($LxY_{X(28)}YxxL$), the tyrosines are separated by 28 amino acids. Based on the low phagocytic activity of C-CaLITR 2.0, I re-examined its tyrosine motifs and their surrounding amino acids. I then concluded that the first tyrosine region closely resembles that of an ITIM ([V/I/S/L]xYxx[L/I/V]), while the second tyrosine region resembles that of an ITSM (TxYxx[V/I/L]). The presence of a classically inhibitory motif (i.e., ITIM) might explain the low phagocytic phenotype (16% of cells show at least one engulfed bead target; see Figure 4.3) by C-CaLITR 2.0. This is consistent with observations made with the

zebrafish (*Danio rerio*) receptor, DrLITR 15.1 (Niemand & Stafford, 2023). This receptor contains a tandem of two ITIMs and an ITSM and showed negligible phagocytic activity compared to IpLITR 2.6b. Another example of a tandem ITIM and ITSM-containing receptor is human G6B-b, which was shown to inhibit thrombocyte aggregation, possibly due to the preferential binding of SHP-1 and/or SHP-2 (Coxon et al., 2012). It is possible that C-CaLITR 2.0 might operate in a similar fashion, where it binds to phosphatases such as SHP-1 and/or SHP-2 and, therefore, inhibits activating molecules (such as Syk, PLCγ, PI3K) in mediating a stimulatory effector function (Huang et al., 2003). This suggested mechanism can be confirmed by examining pSHP-1/pSHP-2 levels in stimulated C-CaLITR 2.0-expressing cells and by examining the protein-to-protein interaction in co-immunoprecipitation assays. In comparison, I also showed that the splice variant C-CaLITR 2.1 induced a significantly higher phagocytic activity than C-CaLITR 2.0 when stimulated by either small or large bead targets (Fig 4.3D and Fig 4.4G). Specifically, the difference is more prominent at the later stages of beads-cell interaction (30-60 minutes) but negligible at an earlier stage (15 minutes). Furthermore, C-CaLITR 2.1 induces a significantly higher phagocytic activity of smaller beads than larger beads. Collectively, these observations indicate a slow-progressing and size-dependent phagocytic phenotype induced by C-CaLITR 2.1.

In terms of the functional difference when compared to C-CaLITR 2.0, this can be attributed to the CYT-splicing event in C-CaLITR 2.1, which removed a 29 amino acid sequence, including an ITIM. While exceptions do exist, it is well established that ITIMs inhibit activating cellular responses, including phagocytosis (Huang et al., 2003). Therefore, it is possible that the removal of the ITIM from C-CaLITR 2.1 prevented the recruitment of inhibitory phosphatases (i.e., SHP-1, SHP-2); allowing C-CaLITR 2.1 to associate with

activating kinases (i.e., Syk, SFKs) and propagate downstream signaling cascades leading to phagocytosis. It is important to note that C-CaLITR 2.1 contains a lone tyrosine-based signalling motif, which is an ITSM. For this reason, the slow-progressing and size-dependent phagocytic activity of C-CaLITR 2.1 can be attributed to that motif. Yet, this conclusion can only be confirmed by mutating the tyrosine in ITSM and examining the mutant's phagocytic activity. However, while signalling motifs containing one tyrosine are not frequently shown to recruit activating molecules and induce stimulatory functions, there are few exceptions in the literature that might suggest a mechanism for CaLITR 2.1-mediated phagocytosis.

Signalling motifs such as the single tyrosine HemITAMs were shown to recruit Syk through dimerization with another identical HemITAM-containing receptor. This way, one SH2 domain of Syk can bind to a phosphorylated HemITAM in one receptor, and the other SH2 domain of Syk will bind to the HemiITAM found in the proximal, identical receptor. Such a mechanism was shown in the C-type lectin receptor, CLEC-2 (Hughes et al., 2010; Mori et al., 2012)). C-CaLITR 2.1 may behave in a similar fashion where stimulation leads to receptor dimerization that creates a perfect binding dock for two SH2 domain-containing molecules such as Syk. This suggested mechanism can be examined by tagging C-CaLITR 2.1 with another epitope (e.g., FLAG), transfecting the FLAG-tagged construct with cells expressing HA-tagged C-CaLITR 2.1. Then, using co-opsonized beads, two C-CaLITR 2.1 proteins can be cross-linked, and pSyk recruitment can be observed in co-IP assays and confocal microscopy of the phagocytic cup during phagocytosis. Alternatively, dimerization of C-CaLITR 2.1 can recruit SHP-2, as in the case of PD-1 (Patsoukis et al., 2020). SHP-2 may also function as a positive regulator of cellular activation pathways (such as ERK pathway), and recent research indicates that SHP-2 removal of phosphate groups from the C-src kinase (Csk) binding protein (Cbp)

impedes the ability of Csk to inhibit Src-family kinases (SFKs) (Zhang et al., 2004). In Chapter V, I showed that C-CaLITR 2.1-mediated phagocytosis is SFK-dependent, so perhaps, the indirect-inhibitory activity of SHP-2 on inhibitors (Csk) of SFK leads to the phagocytic phenotype observed by C-CaLITR 2.1. Overall, my findings in Chapter IV represent the first functional analysis of alternatively spliced immune receptors in teleosts, highlighting a scenario in which the excision of 29 amino acids from the cytoplasmic tail of a receptor resulted in an enhanced phagocytic activation phenotype. Moreover, this study proposes that ITSMs can initiate phagocytosis in AD293 cells, albeit with less efficacy compared to ITAM-mediated phagocytosis, as seen in receptors like C-CaLITR 6.1 and IpLITR 2.6b. Notably, phagocytosis triggered by the ITSM-bearing C-CaLITR 2.1 is characterized by a temporal delay and slower progression, which paves the way for future research into the signalling pathways underlying this distinctive phenotype.

In Chapter V, my objective was to highlight the signalling pathways implicated in the phagocytic phenotype mediated by C-CaLITR 2.1. A pharmacological inhibitors analysis was conducted to investigate the involvement of key signalling molecules and elements (such as Syk, SFKs, PI3K, and F-actin polymerization) commonly associated with classical phagocytic mechanisms in Fc-receptors (Swanson & Hoppe, 2004). Cells expressing C-CaLITR 2.1, 2.0 or 6.1 were pre-treated with a fixed amount of Cytochalasin D (inhibitor of F-actin polymerization), Wortmannin (PI3K inhibitor), PP2 (Src-family kinases inhibitor) and ER 27319 (Syk inhibitor) and phagocytosis assays were performed as described in section 3.4.1. Overall, I showed that C-CaLITR 2.1 mediates phagocytosis through a pathway dependent on actin polymerization, PI3K, and SFKs, and, to a lesser extent, involves the activity of Syk. However, the data for Syk inhibition does not show a strong magnitude of inhibition and requires further examination. In

addition, the signalling profile varies when C-CaLITR 2.1 interacts with beads of different sizes, specifically under PI3K blockage by wortmannin. For instance, phagocytosis of 2 μm beads experienced a non-significant reduction of 41.4% when compared to the vehicle control. In contrast, phagocytosis of larger (4.5 μm) beads led to significant suppression of phagocytic activity by 71.2% (with a p-value of 0.0005 or less), as shown in Figure 5.1C. Notably, this approximate 30% difference in the effectiveness of PI3K inhibition on phagocytosis between smaller and larger beads was uniquely observed for C-CaLITR 2.1. While the findings presented in Chapter V do not identify the specific signalling molecules responsible for C-CaLITR 2.1-mediated phagocytic activity, they provide an important overview of the general pathways utilized by an ITSM-bearing receptor in mediating this universal immune function. Further examination of specific signalling will be needed to untangle the proximal agents driving this unique, slow-progressing, and size-dependent phagocytic phenotype.

6.2 Future Directions

6.2.1 Functional Resolution of Alternatively Spliced CaLITRs through Receptor Cross-linking.

While assays focusing on the activation of a singular type of immune receptor yield valuable insights into cellular signalling modulation, they fall short of capturing the intricacies of the regulatory dynamics that arise from the engagement and subsequent activation of multiple receptors. For instance, professional phagocytes frequently interact with targets that present various ligands, leading to the simultaneous activation of multiple corresponding receptors. This process of integrating proximal submembrane signalling from diverse receptors is known as crosstalk (Freeman & Grinstein, 2014; Underhill & Goodridge, 2012). For example, when TLRs and FcRs are co-engaged, there is a synergistic enhancement in the secretion of pro-

inflammatory cytokines, which is either absent or markedly reduced when these receptors are activated independently (Underhill & Gantner, 2004; Hajishengallis & Lambris, 2016). When FcRL5 and the BCR are crosslinked on the surface of marginal zone B-cells, signalling through the BCR is inhibited; an effect not seen when either receptor was engaged separately (Rostamzadeh et al., 2018). Mechanisms of receptor cross-talk were previously investigated in our lab using the phagocytic receptor IpLITR 2.6b, and the inhibitory receptor IpLITR 1.1b, within the context of the AD293 heterologous expression system (Fei et al., 2020). In this study, AD293 cells were co-transfected with HA-tagged IpLITR 2.6b and FLAG-tagged IpLITR 1.1b. Subsequently, fluorescent bead targets were co-opsonized with anti-HA and anti-FLAG monoclonal antibodies that were introduced to facilitate the co-engagement of both sets of catfish receptors to investigate crosstalk. The ensuing phagocytic response was then quantified utilizing a flow cytometry-based phagocytosis assay. IpLITR 2.6b phagocytic capacity was significantly diminished when cross-linked with IpLITR 1.1b. Most notably, the initiation and sustenance of IpLITR 1.1b inhibitory effects was dependent on specific and distinct proximal or distal CYT regions within IpLITR 1.1b. Specifically, the membrane distal CYT region contained two ITIMs and an ITSM, which were essential in initiating the inhibitory effects. Similar to 1.1b, CaLITR 2.0 contains an ITIM and ITSM, and may potentially inhibit the activity of a stimulatory receptor such as CaLITR 6.1. In addition, CaLITR 2.1 may also behave differently when cross-linked with a stimulatory receptor, mainly due to the presence of its switch motif. SLAM family receptors such as SLAMF4 and SLAMF6, which contain ITSMs in their CYT, were shown to induce either stimulatory or inhibitory signals based on the type of cell they are expressed in, and the adaptor molecules (i.e., SAP or SHP-2) they recruit (Dragovich & Mor, 2018). Both C-CaLITR 2.0 and C-CaLITR 2.1 could be cloned into a vector that tags the N-terminal of the

protein with a distinctive tag, for example the FLAG-tagged CMV-9 vector, to selectively crosslink it with the HA-tagged C-CaLITR 6.1. Indeed, preliminary experiments were conducted to transfect FLAG-tagged C-CaLITR 2.0 and C-CaLITR 2.1, separately, into AD293 cells that already express HA-tagged C-CaLITR 6.1. While expressing 2.0 was difficult, I was able to express both FLAG-tagged C-CaLITR 2.1 and HA-tagged C-CaLITR 6.1 in the same cell population. Co-engagement with co-opsonized 4.5 μm beads led to a slight reduction (10-15%) after 30 minutes of C-CaLITR 6.1-mediated phagocytosis (unpublished data). For future studies, the phagocytosis assays could be extended to 60 minutes. Just as we have seen the slow-progressing, delayed, phagocytic phenotype of C-CaLITR 2.1 when engaged by itself, it could also show a slow-progressing-inhibition phenotype when cross-linked with a stimulatory receptor. Future efforts should also focus on co-expressing FLAG-tagged C-CaLITR 2.0 with HA-tagged C-CaLITR 6.1. Due to the presence of a tandem ITIM and an ITSM in the CYT region of C-CaLITR 2.0, a tandem that was shown to initiate a strong inhibitory effect in IpLITR 1.1b, C-CaLITR 2.0 functional potential may only be fully underst in a receptor cross-talk system. Overall, the cross-talk system can then tell us more about the functional differences attributed to the CYT-splicing event in goldfish immune receptors.

6.2.2 Identification of Specific Signaling Molecules Recruited during C-CaLITR 2.1-mediated Phagocytosis.

The pharmacological studies discussed in Chapter V established a general profile of the signalling molecules and elements that are involved in C-CaLITR 2.1-mediated phagocytosis. I showed that C-CaLITR 2.1 depends on actin polymerization, PI3K, and Src-family kinases to induce its unique, delayed and size-dependent phagocytic phenotype. My findings also hint at some degree of Syk involvement, however, the magnitude of inhibition by ER 27319 (i.e., Syk

inhibitor) was 2x and 3x lower than that of other inhibitors (Cytochalasin D: F-actin polymerization, Wortmannin: PI3K, PP2: SFKs, see Figure 5.1C and 5.1D). One way to verify whether Syk is involved or not is through confocal microscopy imaging. Cells expressing C-CaLITR 2.1 can be stimulated with non-fluorescent bead targets, then fixed at a specific time point (e.g., 8 minutes), followed by bead staining, cell membrane permeabilization, and staining for phosphorylated Syk (pSyk) using fluorescence-conjugated antibodies specific to pSyk. The goal is to visualize sites of target engagement where beads are partially engulfed and monitor for Syk-associated fluorescence at those sites. Previously in our lab, Syk recruitment at the phagocytic cup was observed during phagocytosis mediated by ITAM-bearing IpLITR 2.6b and DrLITR 1.2 (Niemand & Stafford, 2023). Efforts to visualize this phenotype were initiated during C-CaLITR-mediated phagocytosis, as C-CaLITR 6.1 showed hints of Syk recruitment, but nothing was observed for C-CaLITR 2.1. Worth noting that antibody sensitivity and laser settings needed more optimization for obtaining clear images of Syk recruitment. Future experiments should also look at fixing the cells at a later time point than 8 minutes, due to the unique, slow-progressing nature of C-CaLITR 2.1's phagocytic phenotype. Very few phagocytic cups were observed when C-CaLITR 2.1 cells undergoing phagocytosis were fixed at 8 minutes. In addition to Syk, ITSM-containing receptors (i.e., SLAM family genes, PD-1) were shown to recruit Fyn, SAP kinases, SHP-1 or SHP-2 phosphatases (Dragovich & Mor, 2018; Patsoukis et al., 2020). I propose constructing a western blot panel where the aforementioned molecules can be examined in cell lysates from C-CaLITR 2.1 expressing cells after stimulation with bead targets. Cell lysates can be blotted with either: i) α-pSyk, ii) α-pSHP-1, iii) α-pSHP-2, iv) α-pSAP and v) α-pFyn, antibodies. Cell lysates can also be blotted with anti-PI3K antibody to examine if there is a difference in protein level when stimulated with small versus large beads.

Overall, identifying the specific molecules responsible for C-CaLITR 2.1-mediated phagocytic phenotype can be examined using confocal microscopic techniques, and examination of protein levels.

6.2.3 Confirming of the role ITSM in C-CaLITR 2.1-mediated phagocytosis.

Due to the presence of a single tyrosine motif (i.e., ITSM) in the CYT domain of C-CaLITR 2.1, it was reasonable to suggest that this motif is facilitating signal transduction during CaLITR 2.1-mediated phagocytic activity. However, I cannot conclusively state that this phenotype is ITSM mediated without creating a mutant lacking the functional tyrosine (Y^{252}) in the ITSM region. I suggest creating a mutant, such as C-CaLITR 2.1$^{CYT\text{-}ITSM\ -/-}$ with site-directed mutagenesis in which Y^{252} is converted to phenylalanine, F^{252}, as an example. Once this mutant construct is created, it could be examined for its phagocytic activity in AD293 cells, using similar assays performed on C-CaLITR 2.1 $^{CYT\text{-}WT}$ in Chapters IV and V. While this does not align completely with the objective of the thesis, which is to resolve the functional differences due to a CYT-region alternative splicing event, it could very well add to the body of knowledge on how switch motifs can modulate immune effector functions, and to verify if C-CaLITR 2.1 phagocytic activity is indeed ITSM-mediated.

6.3 Concluding Remarks

Mechanisms of immunoregulation are multifaceted and occur at various stages of the immune response cycle. Each of these stages involves distinct regulatory mechanisms that ensure the immune response is effective in defending the body against pathogens, yet balanced enough to prevent excessive or inappropriate responses that could lead to autoimmune diseases or chronic inflammation. Taking a reductional approach, our lab has focused on studying immunoregulation at the level of immune receptors and the underlying signalling molecules

involved in a specific functional output. Using LITRs as a model system to study immunity has allowed us to explore interactions between immune receptors and adaptor signalling molecules, the way they control cell cytotoxicity through traditional and non-traditional inhibitory elements (i.e., ITIMs), as well as their ability to cross-talk to fine-tune a universal immune function in phagocytosis. While first discovered in catfish, *litrs* were also found in zebrafish and goldfish. Within the goldfish repertoire, we identified a unique event of alternative splicing in the CYT region of CaLITR 2.0, creating the splice variant CaLITR 2.1. This event resulted in the deletion of 29 amino acids, removing a tyrosine residue potentially implicated in signalling. This presents another layer of immunoregulation, in which the cell has produced a variant that may fine-tune signaling, and consequently, its functional output. To examine the functional consequences of this splicing event, I used Image-Stream-based Phagocytosis. My results show for the first time that CYT-directed alternative splicing in teleost immunoregulatory receptors can indeed fine-tune an effector immune response. C-CaLITR 2.1 was able to induce a significantly higher phagocytic activity than C-CaLITR 2.0. This phenotype was consistent across different conditions. Overall, findings in this thesis suggest that alternative splicing, a modification done at the mRNA level, is a mechanism through which teleost immunoregulatory receptors can fine-tune their immune-related functions.

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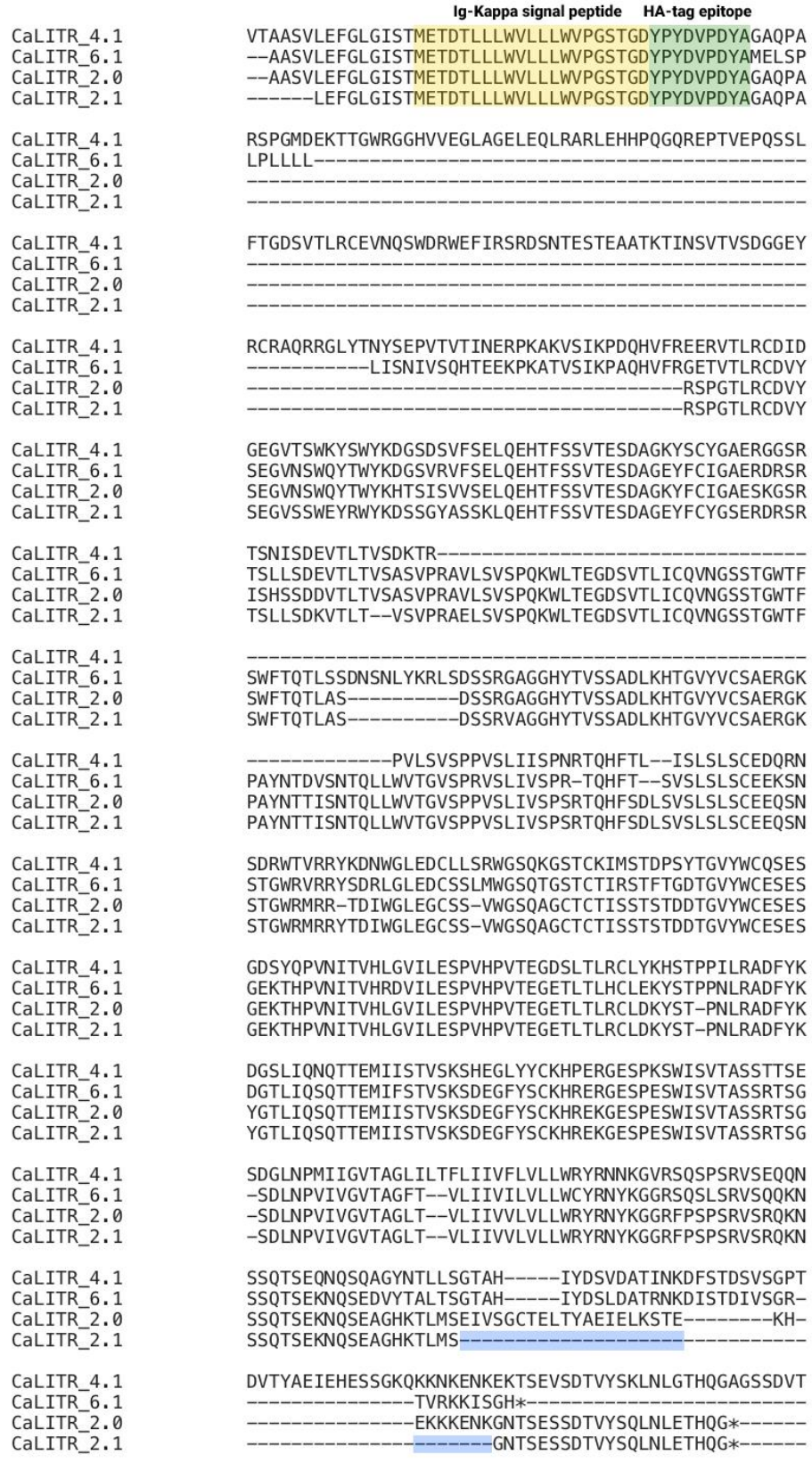
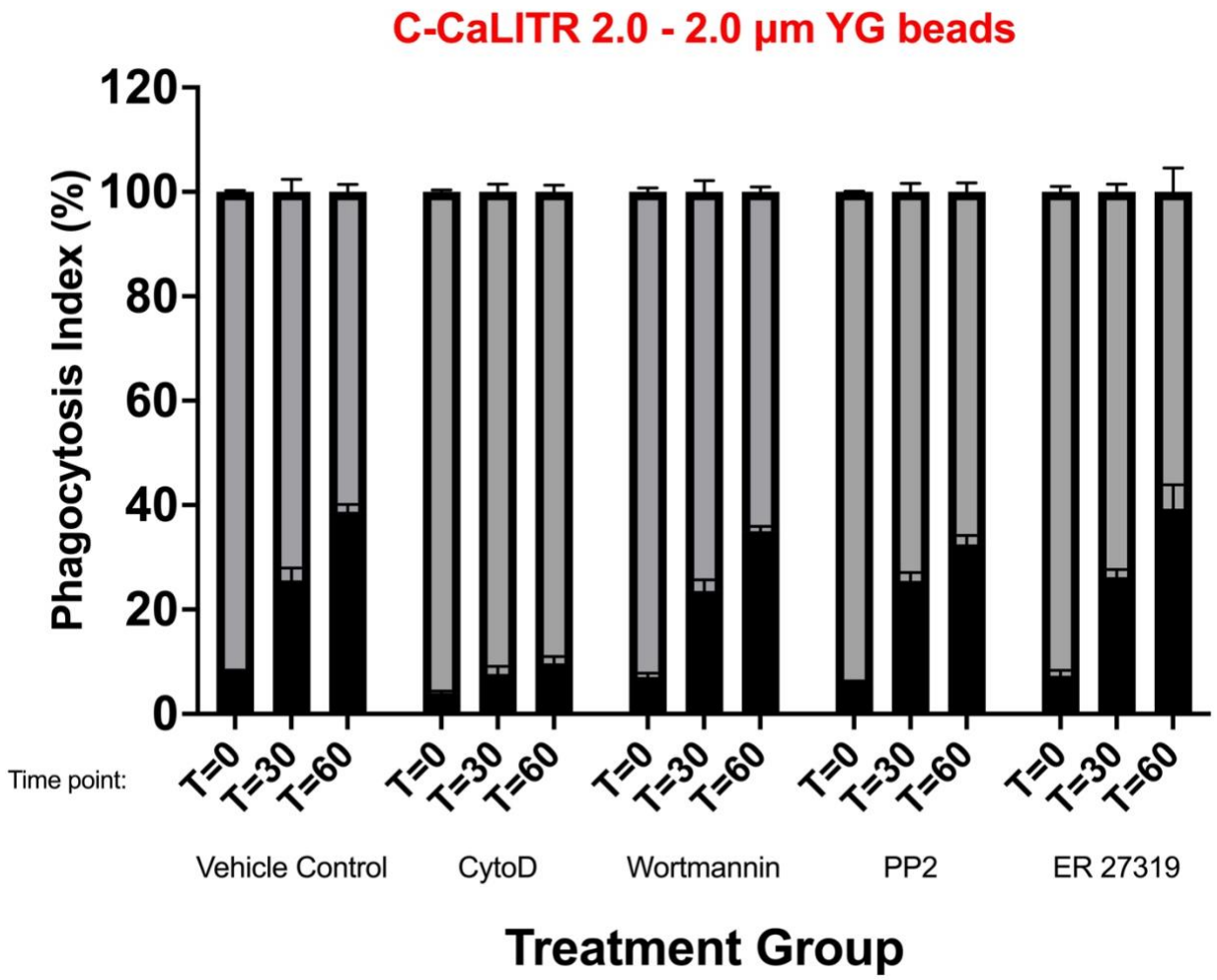
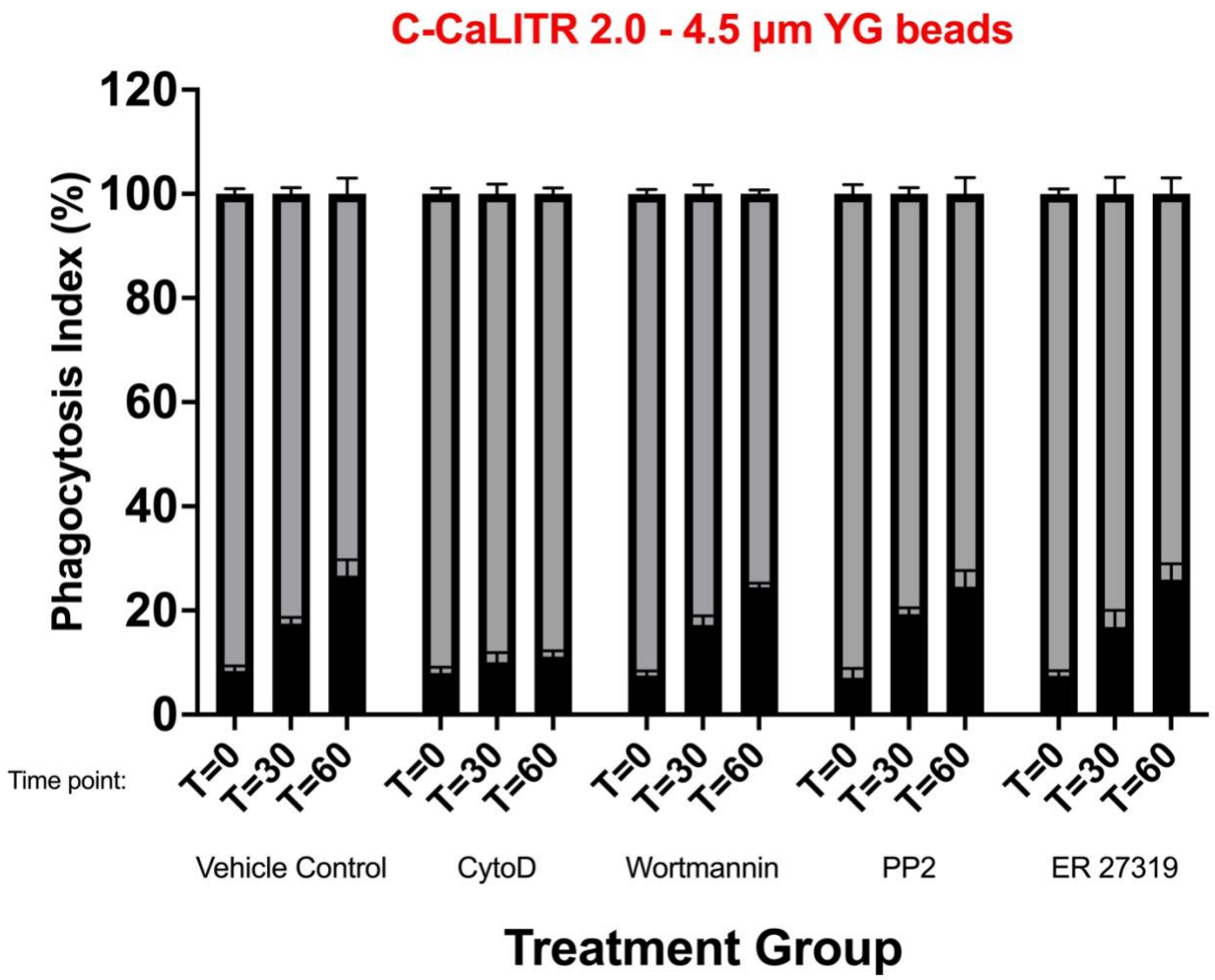
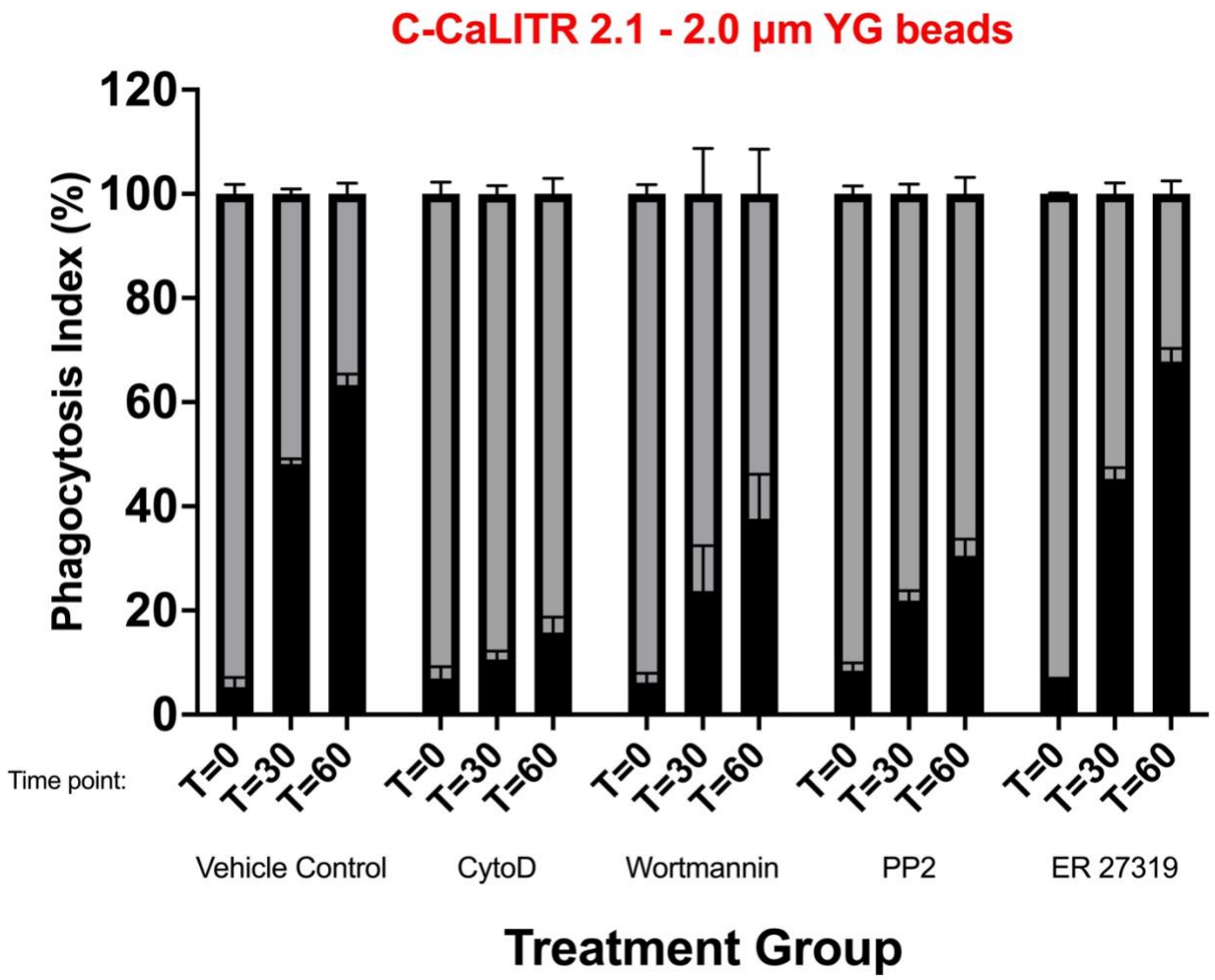
APPENDIX

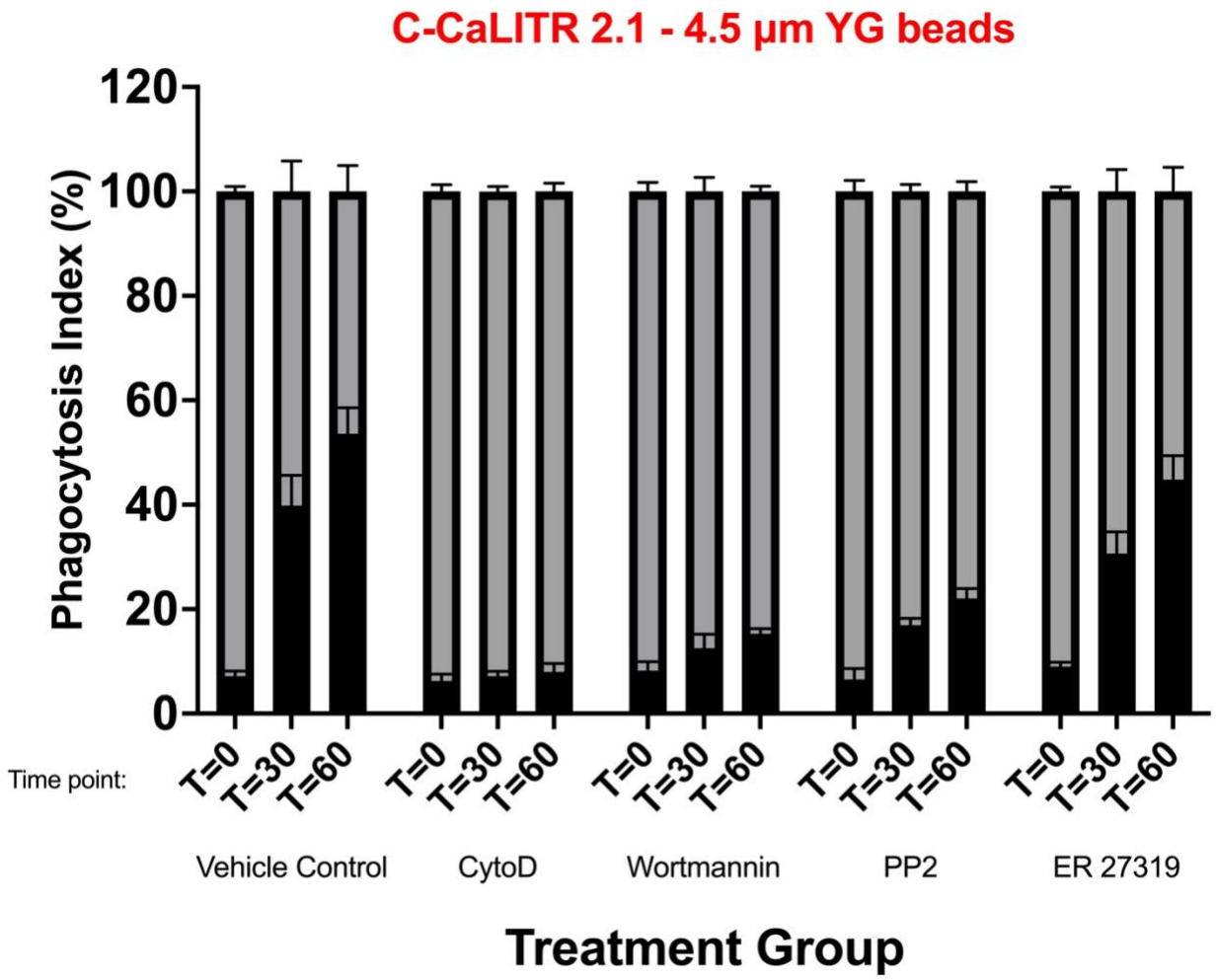

Ig-Kappa signal peptide
HA-tag epitope
CaLITR_4.1 VTAASVLEFGLGISTMETDTLLLWVLLLWVPGSTGDYPYDVPDYAGAQPA
CaLITR_6.1 --AASVLEFGLGISTMETDTLLLWVLLLWVPGSTGDYPYDVPDYAMELSP
CaLITR_2.0 --AASVLEFGLGISTMETDTLLLWVLLLWVPGSTGDYPYDVPDYAGAQPA
CaLITR_2.1 ------LEFGLGISTMETDTLLLWVLLLWVPGSTGDYPYDVPDYAGAQPA
CaLITR_4.1 RSPGMDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREPTVEPQSSL
CaLITR_6.1 LPLLLL----------
CaLITR_2.0 ----------
CaLITR_2.1 ----------
CaLITR_4.1 FTGDSVTLRCEVNQSWDRWEFIRSRDSNTESTEAATKTINSVTVSDGGEY
CaLITR_6.1 ----------
CaLITR_2.0 ----------
CaLITR_2.1 ----------
CaLITR_4.1 RCRAQRRGLYTNYSEPVTVTINERPKAKVSIKPDQHVFREERVTLRCDID
CaLITR_6.1 ----------LISNIVSQHTEEKPKATVSIKPAQHVFRGETVTLRCDVY
CaLITR_2.0 ----------RSPGTLRCDVY
CaLITR_2.1 ----------RSPGTLRCDVY
CaLITR_4.1 GEGVTSWKYSWYKDGSDSVFSELQEHTFSSVTESDAGKYSCYGAERGGSR
CaLITR_6.1 SEGVNSWQYTWYKDGSVRVFSELQEHTFSSVTESDAGEYFCIGAERDRSR
CaLITR_2.0 SEGVNSWQYTWYKHTSISVVSELQEHTFSSVTESDAGKYFCIGAESKGSR
CaLITR_2.1 SEGVSSWEYRWYKDSSGYASSKLQEHTFSSVTESDAGEYFCYGSERDRSR
CaLITR_4.1 TSNISDEVTLTVSDKTR----------
CaLITR_6.1 TSLLSDEVTLTVSASVPRAVLSVSPQKWLTEGDSVTLICQVNGSSTGWTF
CaLITR_2.0 ISHSSDDVTLTVSASVPRAVLSVSPQKWLTEGDSVTLICQVNGSSTGWTF
CaLITR_2.1 TSLLSDKVTLT--VSVPRAELSVSPQKWLTEGDSVTLICQVNGSSTGWTF
CaLITR_4.1 ----------
CaLITR_6.1 SWFTQTLSSDNSNLYKRLSDSSRGAGGHYTVSSADLKHTGVYVCSAERGK
CaLITR_2.0 SWFTQTLAS----------DSSRGAGGHYTVSSADLKHTGVYVCSAERGK
CaLITR_2.1 SWFTQTLAS----------DSSRVAGGHYTVSSADLKHTGVYVCSAERGK
CaLITR_4.1 ----------PVLSVSPPVSLIISPNRTQHFTL--ISLSLSCEDQRN
CaLITR_6.1 PAYNTDVSNTQLLWVTGVSPRVSLIVSPR-TQHFT--SVSLSLSCEEKSN
CaLITR_2.0 PAYNTTISNTQLLWVTGVSPPVSLIVSPSRTQHFSDLSVSLSLSCEEQSN
CaLITR_2.1 PAYNTTISNTQLLWVTGVSPPVSLIVSPSRTQHFSDLSVSLSLSCEEQSN
CaLITR_4.1 SDRWTVRRYKDNWGLEDCLLSRWGSQKGSTCKIMSTDPSYTGVYWCQSES
CaLITR_6.1 STGWRVRRYSDRLGLEDCSSLMWGSQTGSTCTIRSTFTGDTGVYWCESES
CaLITR_2.0 STGWRMRR-TDIWGLEGCSS-VWGSQAGCTCTISSTSTDDTGVYWCESES
CaLITR_2.1 STGWRMRRYTDIWGLEGCSS-VWGSQAGCTCTISSTSTDDTGVYWCESES
CaLITR_4.1 GDSYQPVNITVHLGVILESPVHPVTEGDSLTLRCLYKHSTPPILRADFYK
CaLITR_6.1 GEKTHPVNITVHRDVILESPVHPVTEGETLTLHCLEKYSTPPNLRADFYK
CaLITR_2.0 GEKTHPVNITVHLGVILESPVHPVTEGETLTLRCLDKYST-PNLRADFYK
CaLITR_2.1 GEKTHPVNITVHLGVILESPVHPVTEGETLTLRCLDKYST-PNLRADFYK
CaLITR_4.1 DGSLIQNQTTEMIISTVSKSHEGLYYCKHPERGESPKSWISVTASSTTSE
CaLITR_6.1 DGTLIQSQTTEMIFSTVSKSDEGFYSCKHRERGESPESWISVTASSRTSG
CaLITR_2.0 YGTLIQSQTTEMIISTVSKSDEGFYSCKHREKGESPESWISVTASSRTSG
CaLITR_2.1 YGTLIQSQTTEMIISTVSKSDEGFYSCKHREKGESPESWISVTASSRTSG
CaLITR_4.1 SDGLNPMIIGVTAGLILTFLIIVFLVLLWRYRNNKGVRSQSPSRVSEQQN
CaLITR_6.1 -SDLNPVIVGVTAGFT--VLIIVILVLLWCYRNYKGGRSQSLSRVSQQKN
CaLITR_2.0 -SDLNPVIVGVTAGLT--VLIIVVLVLLWRYRNYKGGRFPSPSRVSRQKN
CaLITR_2.1 -SDLNPVIVGVTAGLT--VLIIVVLVLLWRYRNYKGGRFPSPSRVSRQKN
CaLITR_4.1 SSQTSEQNQSQAGYNTLLSGTAH-----IYDSVDATINKDFSTDSVSGPT
CaLITR_6.1 SSQTSEKNQSEDVYTALTSGTAH-----IYDSLDATRNKDISTDIVSGR-
CaLITR_2.0 SSQTSEKNQSEAGHKTLMSEIVSGCTELTYAEIELKSTE--------KH-
CaLITR_2.1 SSQTSEKNQSEAGHKTLMS----------
CaLITR_4.1 DVTYAEIEHESSGKQKKNKENKEKTSEVSDTVYSKLNLGTHQGAGSSDVT
CaLITR_6.1 ----------TVRKKISGH*----------
CaLITR_2.0 ----------EKKKENKGNTSESSDTVYSQLNLETHQG*------
CaLITR_2.1 ----------GNTSESSDTVYSQLNLETHQG*------

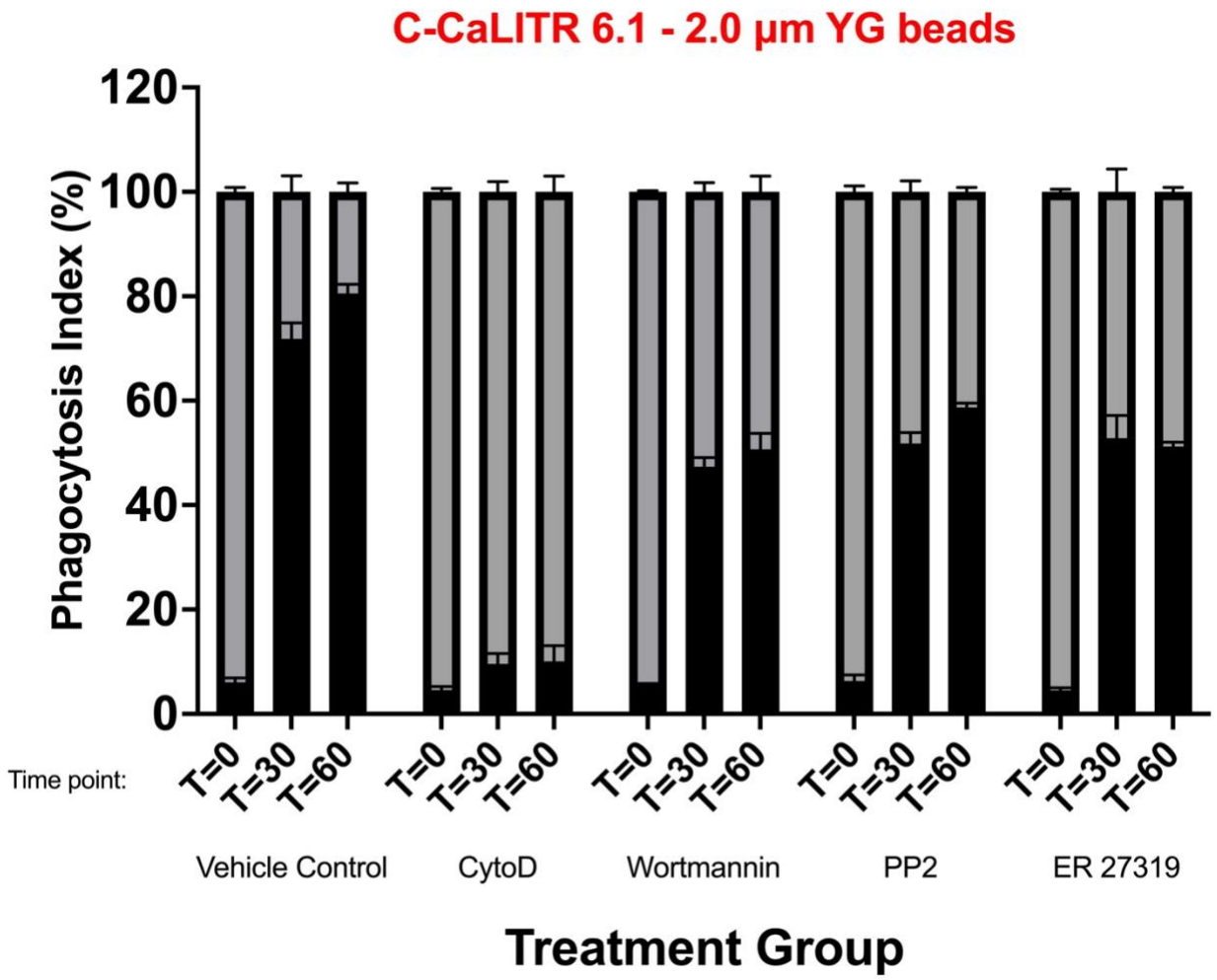
Appendix Figure 1. Predicted Protein Sequences of native CaLITRs. DNA of native *litrs* in pDisplay were sequenced using the pDisplay forward and reverse primers (Table 3.1). Gaps in the alignment are indicated by dashes. Yellow shade indicates the Ig-Kappa leader sequence, while green shade shows the HA-tag. Blue shade indicates the site of the CYT-splicing event in CaLITR 2.1. The Sequence alignment was performed using Clustal Omega.

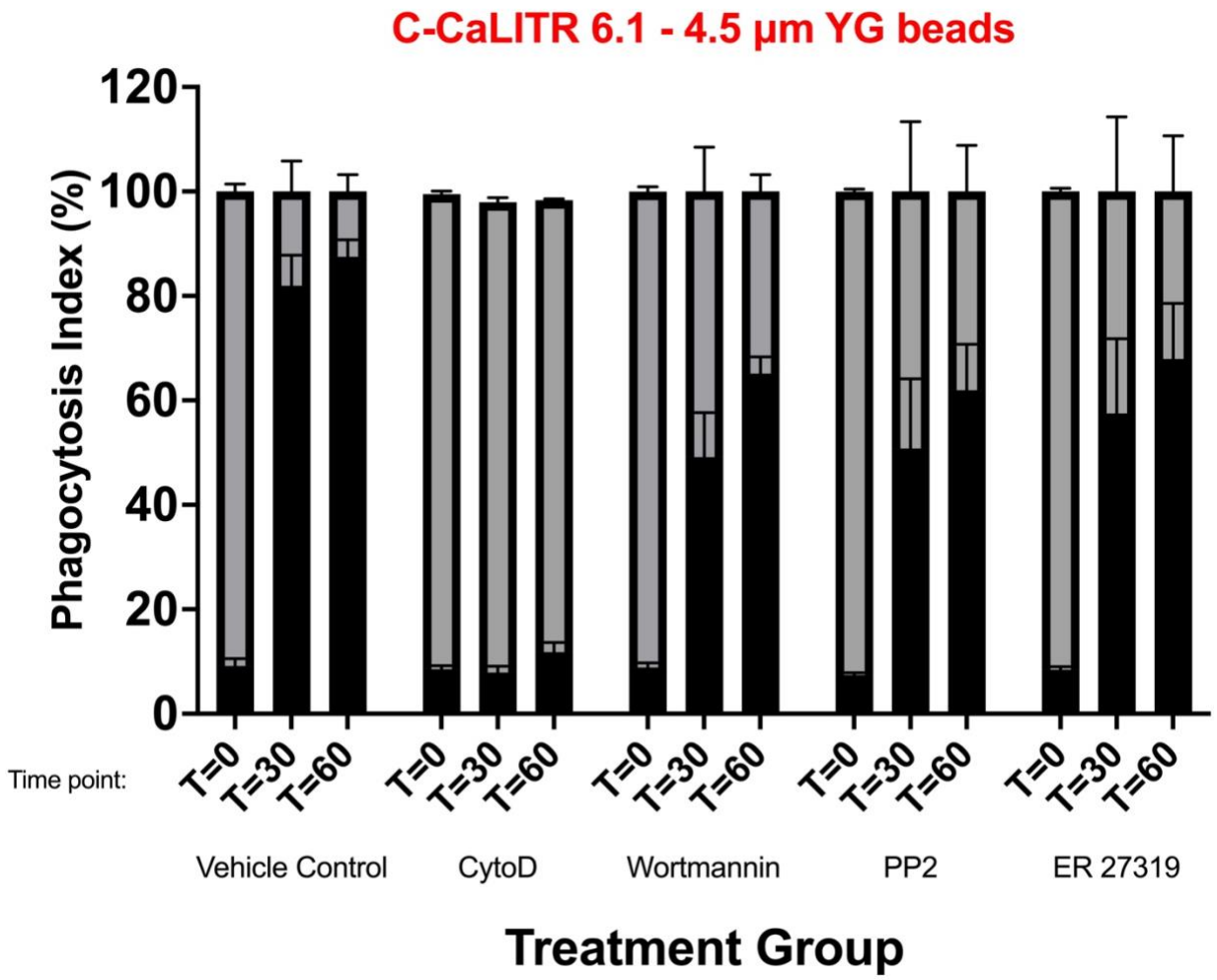

C-CaLITR 2.0 - 2.0 µm YG beads
Phagocytosis Index (%)
120
100
80
60
40
20
0
Time point:
T=0
T=30
T=60
Vehicle Control
CytoD
Wortmannin
PP2
ER 27319
Treatment Group


C-CaLITR 2.0 - 4.5 µm YG beads
Phagocytosis Index (%)
120
100
80
60
40
20
0
Time point:
T=0
T=30
T=60
Vehicle Control
CytoD
Wortmannin
PP2
ER 27319
Treatment Group


C-CaLITR 2.1 - 2.0 µm YG beads
Phagocytosis Index (%)
120
100
80
60
40
20
0
Time point:
T=0
T=30
T=60
Vehicle Control
CytoD
Wortmannin
PP2
ER 27319
Treatment Group


C-CaLITR 2.1 - 4.5 µm YG beads
Phagocytosis Index (%)
120
100
80
60
40
20
0
Time point:
T=0
T=30
T=60
Vehicle Control
CytoD
Wortmannin
PP2
ER 27319
Treatment Group


C-CaLITR 6.1 - 2.0 µm YG beads
Phagocytosis Index (%)
120
100
80
60
40
20
0
Time point:
T=0
T=30
T=60
Vehicle Control
CytoD
Wortmannin
PP2
ER 27319
Treatment Group


C-CaLITR 6.1 - 4.5 µm YG beads
Phagocytosis Index (%)
120
100
80
60
40
20
0
Time point:
T=0
T=30
T=60
Vehicle Control
CytoD
Wortmannin
PP2
ER 27319
Treatment Group

Appendix Figure 2. Phagocytosis of YG beads by C-CaLITR-expressing AD293 cells when pre-treated with Pharmacological inhibitors. 3 x 10^5 AD293 cells expressing C-CaLITR 2.0, 2.1, and 6.1. were pre-treated with pharmacological inhibitors (Table 3.2) and 0.5% DMSO vehicle control for 60 minutes. Without removing drug-containing media, cells were then incubated with either 4.5μm or 2.0 μm YG beads (9 x 10^5), coated in protein G, followed by opsonization with 2 μg/mL αHA mAb, for 60 minutes at 37°C. Samples were then counter-stained and subsequently analyzed using the ImageStream X Mark II. Events were classified as phagocytic (black bars) with at least 1 bead being internalized or surfaced bound (grey bars) with only surface-attached beads. Samples were normalized, and the % values were calculated as # of surface-bound events or # of phagocytic events / # of all bead-associated events. Each bar represents the mean ± SEM of total cells associated with beads from 3 independent experiments.